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EFFICACY OF *MURRAYA KOENIGII* LEAF EXTRACT FOR ATTENUATING THE PROGRESSION OF DIABETIC NEPHROPATHY IN ANIMAL MODELS

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ABSTRACT

Diabetes was induced in male adult albino rats by injecting streptozotocin (65 mg/kg b.wt., i.p.) and left untreated for 9 weeks for the development of nephropathy. Animals were treated with methanolic extract of *Murraya koenigii* (L.) Spreng leaf (MEMK) at two dose levels (200 mg/kg and 400mg/kg) and with standard drug Glimipride (2mg/kg) for 40 days in order to analyse its renoprotective effect, by evaluating metabolic profile, renal function test, electrolyte concentration in blood and urine along with its antioxidant status and histopathological studies. Nephropathy was noted in diabetic rats between 8^{th}-9^{th} week by assessing significant increase in polyuria, proteinuria, serum glucose, albuminuria, creatinine and blood urea nitrogen levels. On 40 days of treatment of MEMK, serum glucose, total cholesterol, HDL- cholesterol, triglyceride including the kidney functions were greatly improved as evidenced by amelioration of blood urea nitrogen, creatinine, serum inulin, total protein concentration, albumin and serum electrolytes (Type IV renal tubular acidosis) and also improvement in antioxidant with decrease in malodialdehyde and increase in catalase, superoxide dismutase and glutathione-s-transferase levels. Histopathological studies of nephropathic animals showed chronic glomerulonephritis with marked improvementin treatment groups. Data from this study suggest that *M. koenigii* (L.) Spreng leaf possesses a protective effect and attenuates the progression of diabetic nephropathy in animals.

KEYWORDS: ***M. koenigii* (L.) Spreng, Diabetic nephropathy, Renal function**

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INTRODUCTION

Diabetic nephropathy (DN) is a serious microvascular complication and 40% of the cases of end stage renal disease (ESRD) are due to this entity. Nephropathy is defined as loss of functions of kidney associated with nephrotic syndrome, glomerulosclerosis, type IV renal tubular acidosis, persistant albuminuria, declining glomerular filteration rate and its associated risk factors are high blood sugar, cholesterol level, proteinuria. The reduced renal function is thus insufficient to support life[1]. It is evident that lipid deposition in the kidneys play an important role in the pathogenesis of diabetic nephropathy in experimental animals, as well as in diabetic patients[2-3]. Recent studies indicate that reactive oxygen species (ROS) play a key role in the pathophysiology of DN[4]. Chronic hyperglycemia, the main initiation and progression of DN, not only generates more reactive oxygen metabolites but also attenuates an antioxidative mechanism through non-enzymatic glycosylation of the antioxidant[5].Indian herbal medicine has been widely used for thousands of years in the diabetes mellitus. The leaves of *M. koenigii* are used in Ayurvedic medicine as anti-diabetic[6] , antioxidant[7] , antimicrobial, anti-inflammatory, hepatoprotective, anti-hypercholesterolemic[8]. The leaves, bark and roots of *M. koenigii* (L.) Spreng. can be used as a tonic and a stomachic[9]. The bark and the roots are used as a stimulant by the physicians[10]. The green leaves are eaten raw for curing dysentery, and the infusion of the washed leaves stops vomiting[11]. Astrong odoriferous oil reported to be found in the leaves and the seeds of *M. koenigii* (L.) Spreng is an essential oil exhibiting a strong antibacterial and antifungal activity[12]. It is reported that girinimbine, a carbazole alkaloid isolated from this plant, inhibited the growth and induced apoptosis in human hepatocellular carcinoma, HepG2 cells[13]. Hence, the objective of the present study is to study the effect of *M. koenigii* L. spreng on attenuating the progression of diabetic nephropathy by evaluating renal function, metabolic profile, management of electrolytes (type IV renal tubular acidosis), renal protein and lipid peroxidation with other renal antioxidant status and morphological changes in the kidney of streptozotocin (STZ) induced DN.

MATERIALS AND METHODS

(i) Plant materials

The leaves of *M. koenigii* L. Spreng (Family: Rutaceae) were collected from the campus of Birla Institute of Technology, Mesra, Ranchi during the month of March-April 2011**.** The plant material was taxonomically identified and authenticated by Dr. K. Karthigeyan, Scientitst 'C', Botanical Survey of India (BSI), Central National Herbarium, Howrah, with ref. no. CNH/103/2011/Tech-II/620. The voucher specimen is stored in the herbarium section of Birla Institute of Technology, Mesra, Ranchi, Department of Pharmaceutical sciences for future reference.

(ii) Preparation of extracts

The leaves of *M. koenigii* L. Spreng were collected and carefully dried in shade for 15 days. To ensure complete dryness, the leaves were kept in hot air oven at 45^0C for 5 minutes. Then, the dried leaves were crushed into coarse powder and stored at room temperature in an air tight container. 50g of this coarse powder was taken and extracted successively in soxhlet apparatus with the series of solvents of increasing polarity as follow: petroleum ether, chloroform and methanol[14]. Each time before extracting with the next solvent, the material was dried. All extracts were filtered through whatman filter paper and concentrated on rotavapor (Buchi Labortechnik AG, CH-9230 Flawil 1/Switzerland) under reduce pressure. To increase the shelf life, the extracts were completely lyophilized by freeze dryer[15] (MPS-55, Korea), yielding 0.6%, 0.9% and 6.0% w/w of petroleum ether, chloroform and methanol and thus methanolic extract of *M. koenigii* L. Spreng obtained were named MEMK selected for in vivo animal models for attenuating the DN in rat.

(iii) Preparation of test samples

The dried MEMK as well as standard drug Glimipride was suspended in 1% w/v carboxymethyl cellulose (CMC) in distilled water prior to oral admintration to the experimental animals. Glimipride IP were received as gift sample from Aristo Pharmaceutical Pvt. Ltd., (Rajsen, Madhya Pradesh).

(iv) Acute toxicity study

Acute toxicity study was carried out for MEMK following OECD 423 guidelines[16]. MEMK was suspended in 1% w/v CMC and was given at a dose of upto 2000 mg/kg body weight p.o. to overnight fasted, healthy mice (n=6). Then the animals were observed for mortality and morbidity for 24 hours. Morbidity like convulsions, tremors, grip strength and pupil dilatation were observed. The animals were observed daily for 14 days.

(v) Experimental design

Inbred male adult albino rats were used for the study and were housed in the Institutional animal house (Reg.no.621/02/ac/CPCSEA) of Birla Institute of Technology, Mesra, Ranchi. All experiments involving animals complies with the ethical standards of animal handling and was approved by the Institutional Animal Ethics Committee (BIT/PH/IAEC/32/2011). All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature, 24-27^{o}C and humidity 60-65% with 12:12 light: dark cycles). The animals were fed either regular diet for good health and diabetogenic diet that induce diabetic phenotype in rat[17]. Regular diet consisting of a mixture of ground whole wheat, normal pellet (Foster Biotech. India, Ltd. Ambala), skimmed milk powder, salts and water *ad libitum*. The diabetogenic diet was given for 7 days to the animals before administration of a single intraperitoneal injection of STZ. It consisted of a mixture of sucrose (50%) w/w, cholesterol (25%) w/w, fat soluble vitamins and tap water *ad libitum*. After occurance of DN in animals after 8-9 weeks, was confirmed by the assessment of various biochemical parameters. After confirmation of DN in animals, they were randomized in the different groups.

Group I- Normal animals received 0.9% w/v saline and 1% w/v CMC daily, p.o., and served as normal control (NC).

Group II – DN animals received normal saline daily, p.o., and served as a DN control.Group III – DN animals received 2mg/kg b.wt , p.o., of Glimipride and served as standard group (TS).

Group IV - DN animals received 200 mg/kg b.wt of methanolic leaf extract of *M. koenigii* L. spreng (MEMK),p.o and served as first test group (T_1MK).

Group V - DN animals received 400 mg/kg b.wt. of methanolic leaf extract of *M. koenigii* L. spreng (MEMK), p.o. , and served as second test group (T_2MK).

The extracts were dissolved in CMC and given orally everyday starting from 0th to 40th day after the induction of DN in rat by force-feeding.

(vi) Preparation of DN animals

The method of Pitchal[18] was used for experimental induction of DN in animals. After acclimatization, overnight fasted animals (given access to drinking water, for 16 hr) were injected bolus of 65 mg/kg body weight STZ, freshly prepared in 3mM citrate buffer (pH 4.5) i.p. The STZ injected animals were then given 5% w/v glucose solution for 5-6 hours following the injection to prevent initial drug induced hypoglycaemic mortality. After 72 hours of STZ injection, blood sugar was estimated by Raptis method[19] and animals having fasting blood sugar above 200 mg/dl was considered to be diabetic. After 3rd, 6th and 9th week, the animals were estimated for the induction of diabetic nephropathy using different renal function test i.e. blood urea nitrogen (BUN) by the method of Sherifa[20], creatinine, albuminuria (UAE) and total protein including blood glucose and body weight according to the method of Muhammad[21]. Nephropathy was noted in animals between 8th - 9th weeks after the administration of STZ (65 mg/kg b.wt, i.p, once) as assessed in terms of above mentioned renal function test and then animals were included for the treatment[22]. Treatment was started from the 0th - 40th day of the experiment.

(vii) Sample collection

Fasting blood sample was collected from the retro-orbital region of the inner canthus of the eye under light ether anesthesia using capillary tubes (Micro Hematocrit capillaries, Mucaps). Serum was separated in a cold centrifuge (Remi, C-24 BL) at 2000 rpm for 10 minute. The serum was estimated for glucose, cholesterol, creatinine, inulin and urea nitrogen levels. The urine sample of animals were collected by keeping the rat in metabolic cages for 24 hours with proper access to drinking water and food and was estimated for clearance of albumin, creatinine, urea and inulin. Upon termination of the studies, animals were anesthetized and blood was obtained from the bifurcation of the aorta for estimation of glycosylated hemoglobin and the animals were sacrificed by exsanguinations. The kidneys were then retrieved and homogenized to prepare the supernatant with which various renal antioxidant studies were carried out. The other kidney was subjected to histopathological studies.

(viii) Biochemical analysis

(A) Metabolic profile

The parameters were analyzed spectrophtometrically including blood glucose based on glucose oxidase method[23], triglycerides[24], total cholesterol[25] and high density lipoprotein based on Chod-PAP method[26]. The kits used for determination of the metabolic profile were obtained from Span diagnostics Ltd. Sachin, Surat, India.

(B) Kidney function tests: It was estimated through the following parameters.

(a) Blood biochemical analysis

The parameters determined were BUN based on modified berthelot method[27], serum creatinine based on Jaffe's kinetic method[28]. The kits used were obtained from Coral, Crest biosystems, Goa, India.

(b) Urine biochemical analysis

Urine volume[29] was measured following by biochemical parameters like UAE using BCG method[30] and proteinuria[31]. The kits used for UAE test were obtained from Autopak®, Siemens medical solutions diagnostics Ltd, Baroda.

(c) Glomerular filtration rate

Clearance is one of fundamental pharmacokinetic parameters. The parameters determined were creatinine clearance[32] and urea clearance[33]. Renal plasma clearance of inulin, C_{inulin}[34] was measured as an index of glomerular filtration rate (GFR). Clearance was calculated using standard clearance formula. Data were normalized for body weight[35]. On 40th day of the experiment, 75mg of inulin was dissolved in saline and were injected i.v. to animal of groups (I-V). Then the volume of urine was measured and analysed for inulin 4-6 times at the mid-point of 20-30 minutes each collection of urine, blood were collected and analyzed for inulin content by the following formula:

C_{inulin} = UV/P, where U= concentration of inulin (mg/100ml), V= concentration of urine (ml/min) and P= concentration of inulin in serum (mg/100ml)

Concentration of inulin was estimated by the method of Joseph[36]. 1 part of serum and 15 part of water were mixed with somogyideproteinizing reagent I (2 part of 10% $ZnSO4.7H_20$ and 2 part of 0.5 N NaOH. These solution neutralized each other precisely by titrate with phenolpthalein as indicator) and then filtered through a whatman filter paper. Then 2ml of filtrate was transfer in a colorimeter tube (1) and in another tube (2) of 2ml of the standard solution containing 0.02 mg per ml of inulin (diluted 1:50). In tube (1) 1ml of resorcinol-thiourea reagent II (0.1gm of resorcinol + 0.25g thiourea + 100 ml of glacial acetic acid mixture) were mixed gently and placed tube in water bath at 80^0C for 10 min. Then both the tubes were removed and kept in the dark to cool down and to complete reaction and finally reading was taken on a colorimeter at 520 mμ. For blank reading was taken at 100 mμ. Concentration of inulin in serum (mg/100ml) were calculated using following formula[37]:

Concentration of inulin in serum (mg/100ml) = (optical density of unknown /optical density of standard) × mg % of inulin per 100 ml of concentration of serum.

Concentration of inulin present in serum was depicted by plotting the graph as concentration of inulin in serum versus time. In case of the measurement of concentration of inulin in urine,

urine was treated with reagent I that removed the proteins. At the same time, tested for its presence by decolorized the urine. Then the filtrate was diluted with distilled water and was treated as outlined above for serum filtrate.

(ix) Serum electrolytes concentrations

Measurement of serum sodium, potassium, chloride, calcium and magnesium (I-V) group were assayed according to method of Varley[38-41] respectively.

(***x) Glycosylated hemoglobin***

Upon termination of the studies, animals were anesthetized, blood was collected from the bifurcation of the aorta for estimation of glycosylated hemoglobin by ion exchange resin method[42] using commercially available kit were obtained from Coral, Crest biosystems, Goa.

(xi) Renal antioxidant status:

After 40 days of treatment, animals were sacrificed by exsanguinations. The kidneys were isolated and their fresh weight was determined gravimetrically and the degree of renal hypertrophy was expressed as the ratio of the weight of the two kidney to total body weight[43]. Then the kidneys were kept at −20 ˚C and subsequently homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). Renal cortical homogenates were centrifuged at 5000 rpm for 10 minutes at 4^{o}C. The resulting supernatant was used for determination of renal antioxidant by determination of malondialdehyde (MDA)[44], superoxide dismutase (SOD)[45], catalase[46] and glutathione s-transferase activities (GST)[47].

(xii) Renal histopathology

Kidney was fixed in 10% formal saline for renal histopathological examination according to method of Aaltonen[48-49].

(xiii) Statistical analysis

Statistical analysis was performed using graph pad prism software version 5.01. The significant differences was analyzed using analysis of variance (ANOVA) followed by Tukey's multiple comparison test (TMCT). All the results were expressed as Mean ± SEM. Unpaired student test was used to compare differences between two groups.

RESULTS

1. Acute toxicity study

Acute toxicity study revealed no mortality or any toxic reactions with oral administration of MEMK even at the highest dose 2000mg/kg b.wt. Hence there were no lethal effects in any of the groups. The biological evaluation of extract is carried out at 1/10th and 1/5th of the highest dose 2000mg/kg b.wt.

2. Induction of DN

Nephropathy was noted in diabetic animals between 8th -9th week after the administration of STZ (65 mg/kg b.wt., i.p, once) as assessed in terms of following different renal function test listed in Table 1. The blood glucose levels were markedly raised (upto 4.5 times) in the diabetic control as compared with normal control on the 3rd, 6th and 9th week of the experiment ($P<0.05$). Diabetic rats exhibited statistically significantly marked polyuria (urine volume), increased UAE level, high serum creatinine as well as BUN and proteinuria ($P < 0.05$). Nodular glomerulosclerosis, hyaline arteriolosclerosis, crescentric glomerulonephritis (GN), membranoproliferative GN with chronic GN was observed by renal histopathological study (Figure 2).Thus nephropathy was induced in diabetic animals. They were distributed into different groups and then chronic treatment was started from the day of induced DN in rats from 0th to 40th day.

3. Metabolic profile

The effect of STZ and the plant extracts on metabolic profile of serum glucose, total cholesterol, HDL - cholesterol and serum triglyceride levels are shown in Table 2. The serum glucose levels were markedly raised in DN as compared with NC on all the days of the experiment ($P < 0.001$). Treatment with T_1MK and T_2MK reduced the serum glucose levels significantly on 40th day as compared to DN

group ($P < 0.05$). The serum cholesterol level was significantly elevated in DN as compared with NC on all days of the experiment ($P < 0.01$). And after treatment with T_1MK and T_2MK, the total cholesterol levels reduced significantly on 40^{th} day as compared to DN group ($P < 0.05$). Decrease in HDL- cholesterol in DN from 0^{th} to 40^{th} day of experiment as compared with NC was noted. In the treatment groups namely T_1MK and T_2MK, there was a significant increase in the HDL- cholesterol levels on 40^{th} day ($P < 0.05$). The serum triglyceride levels were significantly increased in DN as compared with NC from 0^{th} to 40^{th} day of experiment ($P < 0.01$). Treatment with T_1MK and T_2MK reduced the serum triglyceride levels significantly on 40^{th} day as compared to DN group ($P < 0.05$).

4. Body weight

The effect of STZ and the plant extracts on body weight is shown in Table 3. Body weight in DN control and treated groups showed no significant changes as compared to NC group.

5. Kidney function tests

Table 3 summarises the effect of STZ and treatment groups on BUN, serum creatnine and urine volume. The BUN levels were significantly higher in DN as compared to NC ($P < 0.001$). Treatment with T_1MK ($P < 0.01$) and T_2MK ($P < 0.05$) reduced the BUN levels significantly on 40^{th} day as compared to DN group. Creatinine levels became significantly higher in DN compared to NC from 10^{th} day onwards ($P < 0.001$). Treatment with T_1MK and T_2MK reduced the serum creatinine levels significantly on 40^{th} day as compared to DN group ($P < 0.05$). 24 hrs urine volumes was significantly higher ($P < 0.001$) in DN group compared to NC. Treatment with T_1MK ($P < 0.05$) and T_2MK ($P < 0.01$) reduced urine volume significantly as compared to DN group. UAE levels, protein content, creatinine and urea clearance have been tabulated in Table 4. UAE levels became significantly higher from 0^{th} to 40^{th} day in DN compared to NC ($P < 0.001$). Treatment with T_1MK ($P < 0.05$) and T_2MK ($P < 0.01$) reduced the UAE levels significantly on 40^{th} day as compared to DN group. Protein levels became significantly higher in DN compared to NC from 10^{th} day onwards ($P < 0.001$). Treatment with T_1MK ($P < 0.01$) and T_2MK($P < 0.05$) reduced the protein levels significantly on 40^{th} day as compared to DN group. Creatinine clearance decreased significantly in DN group compared to NC from 10^{th} to 40^{th} day. Treatment with T_1MK and T^2MK improved the creatinine clearance significantly as compared to DN group ($P<0.05$). Urea clearance decreased significantly in DN group compared to NC from 10^{th} to 40^{th} day. Treatment with T1MK ($P < 0.01$) and T2MK($P < 0.05$) improved the urea clearance significantly as compared to DN group. The effect of STZ and plant extracts on serum inulin levels in all groups is shown in (Table 5 and Figure 1a) as concentration versus time. Serum inulin levels was significantly increased in DN group as compared to NC. On 40^{th} day treatment showed significant reduction in inulin at the 0^{th} min after the 40^{th} day of experiment ($P < 0.05$). The effect of STZ and plant extracts on inulin clearance (Figure 1b) was statistical significant elevated in DN than the treatment groups as compared to NC ($P < 0.05$).

6. Serum electrolytes concentration

The results of electrolyte profile are shown in Table 6. Sodium, chloride, calcium and magnesium were significantly decreased in DN group as compared to NC ($P < 0.001$). Treatment with T_1MK and T_2MK ($P < 0.05$) showed a significant increase in serum sodium, chloride, calcium and magnesium after 40^{th} day of experiment. Potassium was significantly increased in DN group as compared to NC ($P < 0.001$. Treatment with T_1MK and T_2MK ($P < 0.05$) showed significant decrease in serum potassium after 40^{th} day of treatment.

7. Glycosylated hemoglobin

The effect of STZ and the plant extracts on glycosylated hemoglobin levels is shown in (Table 7). Glycosylated hemoglobin was significantly increased in DN group as compared to NC ($P < 0.01$). T_1MK and T_2MK ($P < 0.05$) showed significantly decreased glycosylated hemoglobin levels after 40^{th} day of treatment.

8. Renal hypertrophy

The effect of STZ and the plant extracts on renal hypertrophy is shown in (Table 6). Kidney weight was significantly increased in DN group as compared to NC ($P < 0.05$). T_1MK and T_2MK ($P < 0.05$) showed significantly decreased kidney weight after 40th day of experiment. Renal hypertrophy was significantly increased in DN group as compared to NC ($P < 0.05$). T_1MK and T_2MK ($P < 0.05$) showed significantly decreased renal hypertrophy after 40th day of treatment.

9. Renal antioxidant status

The effect of STZ and the plant extracts on renal antioxidant status is shown in (Table 6). MDA levels were significantly increased in DN group as compared to NC ($P < 0.01$). T_1MK and T_2MK treatment ($P < 0.05$) significantly decreased MDA levels after 40th day of experiment. GST levels were significantly decreased in DN group as compared to NC ($P < 0.01$). T_1MK and T_2MK ($P < 0.05$) showed significantly increased GST levels after 40th day of experiment. SOD levels were significantly decreased in DN group as compared to NC ($P < 0.01$). T_1MK and T_2MK ($P < 0.05$) showed significantly increased SOD levels after 40th day of experiment. Catalase levels were significantly decreased in DN group as compared to NC ($P < 0.01$). T_1MK and T_2MK ($P < 0.05$) showed significantly increased catalase levels after 40th day of experiment.

10. Renal morphological study

The photomicrographs of the histopathological section of the kidneys were taken in Leica microscope (Model - DME) at a magnification of 40 X and the results are shown in (Figure 2). The histopathological picture of rat kidney showed nodular glomerulosclerosis, hyaline arteriolosclerosis , enlargement of glomeruli, capillary tuft and of subcapsular urinary space, renal damage in DN group. The NC group showed normal glomeruli tufts, any enlarged glomeruli and the subcapsular space was small. On 40th day of treatment with plant extracts the normal glomerulus was seen as compared to the DN group.

Table 1

Estimation of biochemical parameters after induction of diabetes for progression of DN

Group	3rd week		6th week		9th week	
Parameter	NC	DN	NC	DN	NC	DN
Blood glucose (mg/dl)	74.12 ± 6.24	350.1 ± 20.01*	75.63 ± 7.18	387.3 ± 24.16*	74.09 ± 6.15	410.0 ± 28.96*
Body weight (gm)	170.1 ± 12.1	172.6 ± 2.86	171.2 ± 13.21	171.7 ± 1.21	172.34 ± 11.3	170.92±1.10
BUN (mg/dl)	18.14 ± 2.12	29.98 ± 4.72***	17.60 ± 3.02	31.76 ± 3.15***	17.92 ± 9.96	33.81±3.96***
Serum creatinine (mg/dl)	0.32 ± 1.7	0.36 ± 0.06	0.38 ± 0.6	0.42 ± 3.2**	0.36 ± 0.15	0.58 ± 1.2**
Urine volume (ml/24hrs)	1.80 ± 0.2	9.91 ± 3.1*	1.89 ± 0.3	12.01 ± 4.2*	1.91 ± 0.2	15.02 ± 2.8*
UAE (µg/24hrs)	363.0 ± 22.1	628.0 ± 73.8*	366.2 ± 22.3	1108.2 ± 62.9*	369.0 ± 23.5	1194.8±63.7*
Protein in urine (g/100ml)	0.216 ± 0.02	0.392 ± 0.02**	0.223 ± 0.01	0.220 ± 0.03*	0.220 ± 0.03	0.820±0.03*
Urea clearance (ml/24 hr)	0.7 ± 0.02	0.25 ± 0.05**	0.7 ± 0.01	0.27 ± 0.06**	0.8 ± 0.03	0.30 ± 0.07**

Values are given as mean ± SEM for groups of 8 animals each. DN as compared with the NC groups at the corresponding time interval. Values are statistical significant at *P < 0.05, **P < 0.01 and ***P < 0.001. Abbrevations- NC: normal control, DN: diabetic nephropathic control

Table 2

Effect of 40 days treatment of MEMK at doses of 200mg/kg and 400 mg/kg b. wt. on serum glucose (mg/dl), total cholesterol (mg/dl), HDL- cholesterol (mg/dl) and serum triglyceride (mg/dl) in DN animals

Parameter	Group	0th day	10th day	20th day	30th day	40th day
Serum Glucose	NC	75.12 ± 6.2	74.65 ± 10.3	76.92 ± 6.2	79.30 ± 11.6	77.28 ± 10.7
	DN	402.01 ± 27.8[a]	403.06 ± 31.2[a]	405.90 ± 30.3[a]	404.70 ± 29.6[a]	405.20 ± 28.3[a]
	TS	416.02 ± 26.1[b]	370.04 ± 12.1[b]	307.20 ± 12.0[b]	254.10 ± 10.6[b]	166.01 ± 9.2[b]
	T_1MK	401.31 ± 16.1[c]	391.30 ± 21.0[c]	373.02 ± 12.3[c]	314.41 ± 13.4[c]	250.11 ± 15.4[c]
	T_2MK	400.70 ± 20.0[c]	366.61 ± 18.6[c]	344.10 ± 20.2[c]	290.13 ± 16.7[c]	240.21 ± 16.7[c]
Total Cholesterol	NC	138.81 ± 1.2	140.30 ± 1.3	141.62 ± 1.2	142.31 ± 0.9	141.03 ± 1.0
	DN	266.34 ± 2.6[b]	270.51 ± 3.9[b]	274.03 ± 2.4[b]	280.62 ± 3.4[b]	278.05 ± 2.6[b]
	TS	265.70 ± 3.4[c]	216.81 ± 2.7[c]	200.61 ± 2.9[c]	156.04 ± 3.6[c]	140.27 ± 2.9[c]
	T_1MK	267.11 ± 3.1[c]	255.23 ± 1.5[c]	230.16 ± 2.3[c]	202.32 ± 1.3[c]	170.52 ± 1.8[c]
	T_2MK	265.24 ± 2.5[c]	242.32 ± 3.4[c]	216.84 ± 1.9[c]	192.40 ± 2.3[c]	154.25 ± 2.7[c]
HDL-Cholesterol	NC	41.21 ± 1.2	41.73 ± 1.0	43.90 ± 0.9	42.31 ± 1.0	44.02 ± 1.3
	DN	41.62 ± 2.9[c]	41.12 ± 1.3[c]	40.07 ± 1.8[c]	38.91 ± 1.0[c]	35.34 ± 2.6[c]
	TS	39.23 ± 1.6[c]	42.31 ± 2.4[c]	42.72 ± 1.9[c]	43.14 ± 3.2[c]	44.50 ± 3.4[c]
	T_1MK	37.41 ± 2.4[c]	38.26 ± 2.2[c]	39.23 ± 1.3[c]	40.41 ± 2.1[c]	40.91 ± 2.8[c]
	T_2MK	38.11 ± 2.1[c]	40.05 ± 2.8[c]	41.23 ± 2.5[c]	42.44 ± 2.0[c]	44.03 ± 2.3[c]
Serum Triglyceride	NC	81.31 ± 1.9	81.39 ± 2.6	80.43 ± 0.9	82.17 ± 1.4	79.04 ± 3.2
	DN	175.10 ± 3.4[b]	176.23. ± 1.9[b]	178.71 ± 2.4[b]	180.32 ± 3.2[b]	186.95 ± 1.2[b]
	TS	179.42 ± 2.4[c]	134.34 ± 1.6[c]	96.76 ± 2.5[c]	92.02 ± 3.6[c]	92.73 ± 1.7[c]
	T_1MK	182.51 ± 2.1[c]	135.03 ± 2.5[c]	110.32 ± 1.7[c]	103.64 ± 3.0[c]	101.62 ± 2.0[c]
	T_2MK	181.32 ± 2.3[c]	124.41 ± 2.7[c]	100.45 ± 2.3[c]	99.30 ± 1.8[c]	98.42 ± 1.3[c]

Values are given as mean ± SEM, n = 6. DN control was compared with the NC groups and treatment groups was compared with the DN control groups on 0th, 10th, 20th, 30th and 40th day. [a]Values are statistically significant at P < 0.001, [b]Values are statistically significant at P < 0.01 and [c]Values are statistically significant at P < 0.05.

Table 3

Effect of 40 days treatment of MEMK at doses of 200mg/kg and 400 mg/kg b. wt. on body weight (gm), BUN (mg/dl), serum creatinine (mg/dl) and urine volume (ml) in DN animals

Parameter	Group	0th day	10th day	20th day	30th day	40th day
Body Weight	NC	172.31 ± 10.1	173.73 ± 10.6	174.60 ± 9.6	175.90 ± 12.9	176.81 ± 12.1
	DN	170.61 ± 9.4	170.62 ± 12.3	170.40 ± 10.4	169.90 ± 9.7	169.72 ± 11.3
	TS	174.92 ± 7.2	175.70 ± 3.4	176.41 ± 4.9	179.73 ± 10.6	177.30 ± 7.2
	T_1MK	173.12 ± 4.1	174.30 ± 5.3	174.90 ± 7.0	176.04 ± 3.9	177.16 ± 9.0
	T_2MK	173.79 ± 8.0	174. 82 ± 5.6	175.91 ± 9.3	175.07 ± 9.7	176.24 ± 3.6
BUN	NC	18.41 ± 2.6	18.70 ± 2.6	18.55 ± 2.5	20.23 ± 2.7	23.94 ± 2.4
	DN	33.10 ± 3.9[a]	34.84 ± 3.9[a]	42.62 ± 3.1[a]	43.74 ± 3.9[a]	48.15 ± 4.0[a]
	TS	33.02 ± 3.9[b]	31.61 ± 3.8[b]	29.90 ± 3.7[b]	25.72 ± 3.2[b]	23.03 ± 2.9[b]
	T_1MK	33.11 ± 3.7[b]	33.03 ± 3.9[b]	31.95 ± 3.7[b]	29.92 ± 3.8[b]	28.80 ± 3.7[b]
	T_2MK	32.91 ± 3.8[c]	31.80 ± 3.7[c]	29.66 ± 3.6[c]	28.04 ± 3.5[c]	26.94 ± 3.9[c]
Serum Creatinine	NC	0.33 ± 0.06	0.35 ± 0.05	0.32 ± 0.06	0.31 ± 0.02	0.34 ± 0.03
	DN	0.62 ± 0.09[a]	0.63 ± 0.06[a]	0.65 ± 0.10[a]	0.64 ± 0.09[a]	0.66 ± 0.08[a]
	TS	0.61 ± 0.08[b]	0.56 ± 0.06[b]	0.51 ± 0.04[b]	0.49 ± 0.05[b]	0.48 ± 0.03[b]
	T_1MK	0.62 ± 0.08[c]	0.61 ± 0.11[c]	0.59 ± 0.10[c]	0.58 ± 0.06[c]	0.56 ± 0.09[c]
	T_2MK	0.62 ± 0.07[c]	0.60 ± 0.12[c]	0.59 ± 0.08[c]	0.57 ± 0.08[c]	0.52 ± 0.06[c]
Urine Volume	NC	1.83 ± 0.3	1.92 ± 0.2	2.13 ± 0.0	2.02 ± 0.0	2.09 ± 0.1
	DN	15.10 ± 0.2[a]	15.19 ± 0.1[a]	16.13 ± 0.1[a]	19.12 ± 1.0[a]	19.84 ± 1.5[a]
	TS	15.01 ± 0.1[b]	14.63 ± 0.3[b]	14.14 ± 0.6[b]	14.01 ± 2.3[b]	13.10 ± 3.1[b]
	T_1MK	16.03 ± 0.1[c]	15.03 ± 0.6[c]	14.91 ± 0.4[c]	14.56 ± 1.2[c]	14.70 ± 0.2[c]
	T_2MK	16.00 ± 0.2[b]	14.9 ± 0.6[b]	14.64 ± 0.2[b]	14.20 ± 0.5[b]	13.91 ± 0.9[b]

Values are given as mean ± SEM, n = 6. DN control was compared with the NC groups and treatment groups was compared with the DN control groups on 0th, 10th, 20th, 30th and 40th day. [a]Values are statistically significant at P < 0.001, [b]Values are statistically significant at P < 0.01 and [c]Values are statistically significant at P < 0.05

Table 4

Effect of 40 days treatment of MEMK at doses of 200mg/kg and 400 mg/kg b. wt. on UAE (µg/24 hr), total protein in urine (g/100 ml), creatinine clearance (ml/24 hr) and urea clearance (ml/24 hr) in DN animals

Parameter	Group	0th day	10th day	20th day	30th day	40th day
UAE	NC	364.6 ± 11.4	363.6 ± 20.9	367.7 ± 22.5	369.6 ± 23.5	372.8 ± 19.4
	DN	1196.7 ± 64.8[a]	1212.6 ± 76.7[a]	1284.9 ± 84.2[a]	1364.3 ± 72.9[a]	1396.0 ± 89.3[a]
	TS	1094.8 ± 29.6[c]	1066.7 ± 34.7[b]	1022.0 ± 43.8[b]	939.4 ± 30.0[b]	934.6 ± 48.7[b]
	T_1MK	1195.0 ± 41.0[c]	1190.2 ± 54.2[c]	1183.1 ± 41.8[c]	1162.7 ± 54.1[c]	1120.0 ± 40.2[c]
	T_2MK	1194.9 ± 32.4[b]	1182.4 ± 42.2[b]	1160.0 ± 30.1[b]	1134.7 ± 33.0[b]	1108.0 ± 50.3[b]
Total Protein	NC	0.214 ± 0.01	0.215 ± 0.03	0.217 ± 0.03	0.211 ± 0.04	0.219 ± 0.03
	DN	1.016 ± 0.03[a]	1.204 ± 0.09[a]	1.500 ± 0.84[a]	1.822 ± 0.02[a]	2.001 ± 0.03[a]
	TS	0.295 ± 0.01[b]	0.292 ± 0.05[b]	0.284 ± 0.01[b]	0.283 ± 0.03[b]	0.240 ± 0.04[b]
	T_1MK	0.612 ± 0.01[b]	0.576 ± 0.03[b]	0.510 ± 0.09[b]	0.368 ± 0.02[b]	0.310 ± 0.04[b]
	T_2MK	0.570 ± 0.02[c]	0.480 ± 0.06[c]	0.430 ± 0.04[c]	0.356 ± 0.08[c]	0.260 ± 0.09[c]
Creatinine Clearance	NC	1.41 ± 0.21	1.30 ± 0.16	1.42 ± 0.12	1.32 ± 0.17	1.13 ± 0.09
	DN	1.10 ± 0.01[a]	0.83 ± 0.04[a]	0.64 ± 0.03[a]	0.57 ± 0.01[a]	0.32 ± 0.10[a]
	TS	0.91 ± 0.11[b]	1.01 ± 0.12[b]	1.24 ± 0.02[b]	1.32 ± 0.01[b]	1.39 ± 0.03[b]
	T_1MK	0.62 ± 0.09[b]	0.72 ± 0.07[b]	0.81 ± 0.10[b]	0.93 ± 0.08[b]	1.03 ± 0.06[b]
	T_2MK	0.52 ± 0.11[b]	0.73 ± 0.12[b]	0.87 ± 0.02[b]	1.05 ± 0.05[b]	1.20 ± 0.03[b]
Urea Clearance	NC	0.63 ± 0.02	0.70 ± 0.05	0.81 ± 0.03	0.90 ± 0.02	0.81 ± 0.08
	DN	0.32 ± 0.10[a]	0.29 ± 0.12[a]	0.23 ± 0.09[a]	0.21 ± 0.06[a]	0.19 ± 0.05[a]
	TS	0.34 ± 0.02[b]	0.37 ± 0.16[b]	0.41 ± 0.20[b]	0.49 ± 0.09[b]	0.52 ± 0.14[b]
	T_1MK	0.36 ± 0.04[c]	0.45 ± 0.08[c]	0.48 ± 0.11[c]	0.51 ± 0.07[c]	0.57 ± 0.06[c]
	T_2MK	0.25 ± 0.13[b]	0.38 ± 0.08[b]	0.46 ± 0.04[b]	0.58 ± 0.13[b]	0.60 ± 0.05[b]

Values are given as mean ± SEM, n = 6. DN control was compared with the NC groups and treatment groups was compared with the DN control groups on 0th, 10th, 20th, 30th and 40th day. [a]Values are statistically significant at P < 0.001, [b]Values are statistically significant at P < 0.01 and [c]Values are statistically significant at P < 0.05.

Table 5

Effect of 40 days treatment of MEMK at doses of 200 mg/kg and 400 mg/kg b. wt. on serum inulin levels (mg/100 ml) in DN animals

Group	0th min	25th min	50th min	100th – 400th min
NC	45.61 ± 2.6	26.30 ± 3.2	1.92 ± 1.9	-
DN	70.12 ± 1.2[a]	36.90 ± 2.6[a]	24.21 ± 3.1[a]	13.34 ± 2.8[a]
TS	58.03 ± 2.6[a]	34.80 ± 5.0[a]	20.23 ± 4.4[a]	10.01 ± 6.7[a]
T_1MK	60.34 ± 4.0[a]	49.33 ± 1.9[a]	34.32 ± 2.3[a]	21.68 ± 6.2[a]
T_2MK	50.98 ± 4.3[a]	32.06 ± 3.5[a]	1.97 ± 5.9[a]	-

Values are given as mean ± SEM. [a]Values are statistically significant at P < 0.05 as compared to NC and DN

Table 6

Effect of 40 days treatment of MEMK at doses of 200 mg/kg and 400 mg/kg b. wt. on serum electrolytes concentration, renal hypertrophy (gm) and renal antioxidant status in DN animals

Parameters	NC	DN	TS	T_1MK	T_2MK
Sodium (meq/L)	160.12 ± 0.16	145.23 ± 0.52[a]	159.02 ± 0.62[b]	142.13 ± 0.34[c]	154.11 ± 0.28[c]
Potassium (meq/L)	5.32 ± 0.02	7.12 ± 0.03[a]	5.41 ± 0.02[b]	6.21 ± 0.03[c]	5.50 ± 0.07[c]
Chloride (meq/L)	98.02 ± 0.24	79.13 ± 0.19[a]	81.04 ± 0.26[b]	86.46 ± 0.13[c]	91.38 ± 0.27[c]
Calcium (meq/L)	9.21 ± 0.08	7.61 ± 0.10[a]	10.03 ± 0.12[b]	8.29 ± 0.04[c]	10.40 ± 0.02[c]
Magnesium (meq/L)	4.39 ± 0.09	1.79 ± 0.02[a]	4.12 ± 0.21[b]	2.86 ± 0.11[c]	3.76 ± 0.14[c]
Two kidney weight	1.08 ± 0.01	1.92 ± 0.02[c]	1.35 ± 0.004[c]	1.47 ± 0.02[c]	1.34 ± 0.04[c]
Renal hypertrophy	0.004 ± 0.01	0.010 ± 0.001[c]	0.004 ± 0.02[c]	0.008 ± 0.01[c]	0.006 ± 0.02[c]
MDA(µM/mg of renal)	1.25 ± 0.02	4.12 ± 0.31[a]	1.60 ± 0.01[b]	3.71 ± 0.01[c]	1.89 ± 0.01[c]
SOD(EU/mg protein)	53.7 ± 0.61	29.16 ± 0.41[a]	51.7 ± 0.03[b]	46.3 ± 0.03[c]	50.1 ± 0.07[c]
Catalase (µM/min/mg)	30.01 ± 0.12	14.03 ± 0.26[a]	29.05 ± 0.16[b]	25.4 ± 0.09[c]	27.0 ± 0.11[c]
GST(nM/mg protein)	0.24 ± 0.01	0.05 ± 0.0[a]	0.23 ± 0.0[b]	0.20 ± 0.03[c]	0.22 ± 0.04[c]

Values are given as mean ± SEM. DN control was compared with the NC groups and treatment groups was compared with the DN control groups on 40th day. [a]Values are statistically significant at P < 0.001 [b]Values are statistically significant at P < 0.01 and [c]Values are statistically significant at P < 0.05.

Table 7

Effect of 40 days treatment of MEMK at doses of 200mg/kg and 400 mg/kg b. wt. on glycosylated hemoglobin A1 (% $GHbA_1$), glycosylated hemoglobin A1C (% HbA1C) and mean blood glucose (mg/dl MBG) levels in DN animals

NC			DN			TS			T_1MK			T_2MK		
$GHbA_1$	HbA_1C	MBG	$GHbA_1$	HbA_1C	MBG	$GHbA_1$	HbA_1C	MBG	$GHbA_1$	HbA_1C	MBG	$GHbA_1$	HbA_1C	MBG
6.7	4.88	77.01	18.5[a]	14.77[b]	405.8[b]	9.9[c]	7.56[c]	166.7[c]	12.7[c]	9.91[c]	243.8[c]	9.9[c]	7.56[c]	166.4[c]

DN control was compared with the NC groups and treatment groups was compared with the DN control groups on 40th day. [a]Values are statistically significant at P < 0.001, [b]Values are statistically significant at P < 0.01 and [c]Values are statistically significant at P < 0.05.

Serum inulin levels (mg/100 ml) in DN animals

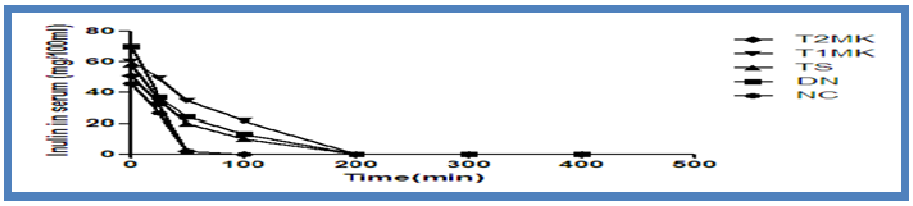
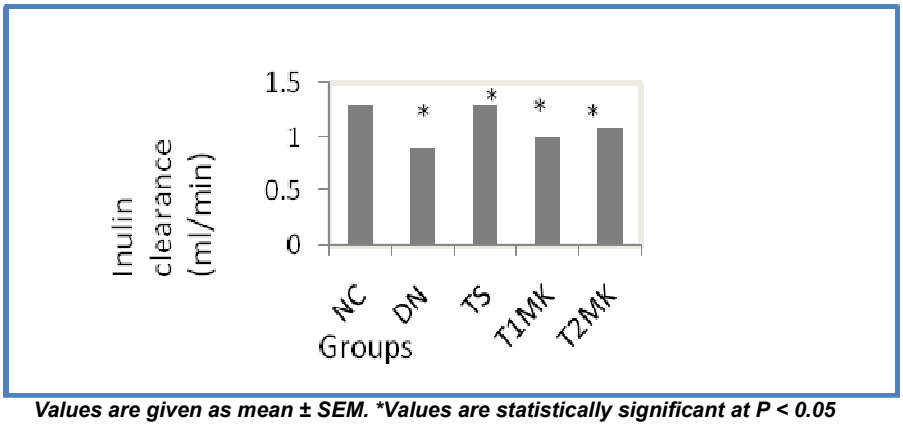


Figure 1a

Effect of 40 days treatment of MEMK at doses of 200 mg/kg and 400 mg/kg b. wt. on serum inulin levels (mg/100 ml) in DN animals

Inulin clearance levels (ml/min per 100g of initial body weight) in DN animals



*Values are given as mean ± SEM. *Values are statistically significant at P < 0.05*

Figure 1b

Effect of 40 days treatment of MEMK at doses of 200mg/kg and 400 mg/kg b. wt. on inulin clearance levels (ml/min per 100g of initial body weight) in DN animals

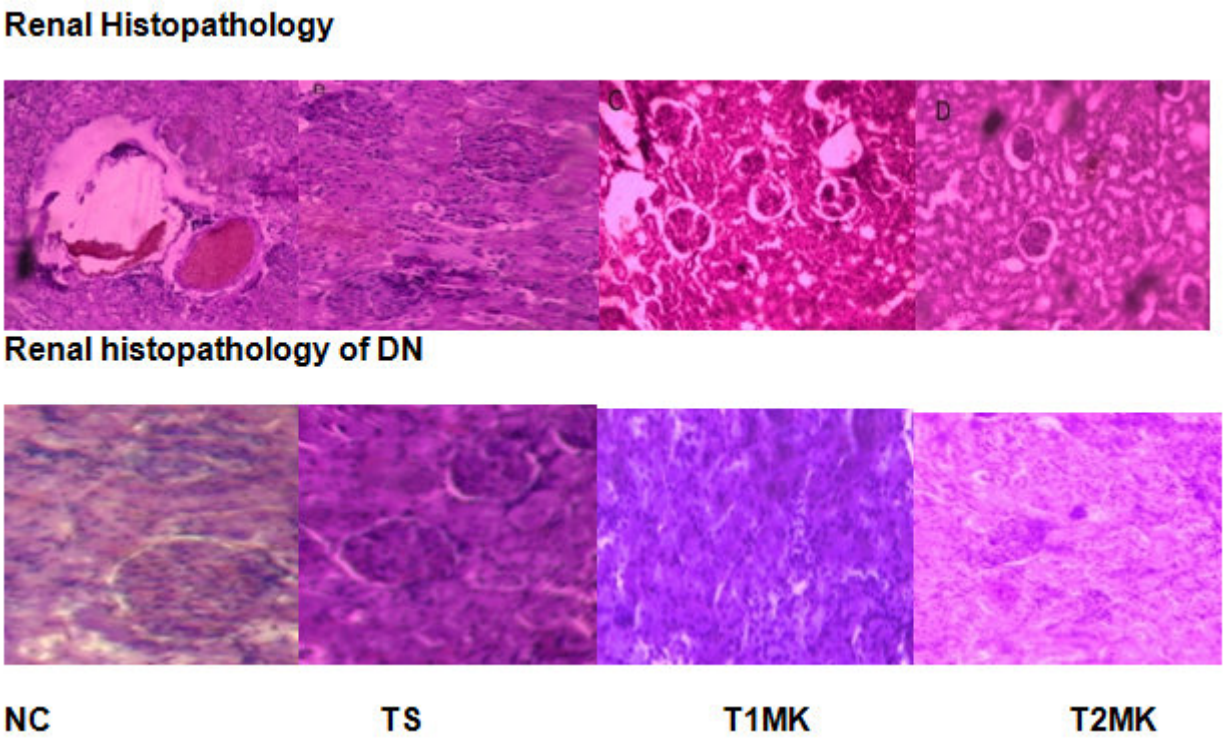


Figure 2
Effect of 40 days treatment of MEMK at doses of 200mg/kg and 400 mg/kg b. wt on renal morphology in diabetic nephropathic animals

DISCUSSION

Traditional medicine has a long history to treat both diabetes and renal diseases. In this study, we investigated the protective effect of *M. koenigii* L. Spreng leaves, a medicine recipe, against DN in diabetic animal models. In present study, diabetic animal model with DN was successfully established by diabetogenic diet (for 7 days diet) and STZ injection[51]. Eight weeks later, diabetic animals developed DN showing typical disorder features which was the same as the previous reported studies (Arulselvan et al., 2006) such as nodular glomerulosclerosis, hyaline arteriolosclerosis, crescentric glomerulonephritis (GN), membranoproliferative GN with chronic GN, glomerular and renal hypertrophy, and extracellular matrix expansion. Severe albuminuria, proteinuria as well as increased creatinine and urea indicated impaired renal function in the diabetic animals[52]. Both hyperglycemia and hyperlipidemia have been linked to diabetic complications, especially renal damage[53-54]. The DN animals in this study demonstrated high blood glucose, increased triglyceride and total cholesterol and decreased HDL- cholesterol as compared with normal control animals. The effect of MEMK on the abnormal level of blood glucose and lipids were evaluated further. Treatment with MEMK reduced the level of the total cholesterol, and serum triglyceride and increased HDL-cholesterol in DN animals. Several studies demonstrated that insulin deficiency or resistance may be responsible for dyslipidemia because insulin has an inhibitory action on HMG-CoA reductase, a key rate limiting enzyme responsible for the metabolism of cholesterol rich LDL particles[55]. Acute insulin deficiency initially causes an increased in free fatty acid mobilization from adipose tissue. This results in increase production of cholesterol rich LDL particle[56].It has been reported that

increased catabolic reactions leading to muscle wasting might be the cause for the reduced weight gain in the diabetic nephropathic animals[57]. An increase in the body weight of DN animals might be due to an improvement in insulin secretion and glycemic control[58]. In the present study, body weight in DN control and treated groups showed no significant changes as compared to NC group. DN is characterized by increase of urine protein and it is a key component of DN[59]. Many studies have shown that the magnitude of urine protein levels is associated with a graded increase in the risk of progression to end stage renal disease and cardiovascular events[60]. Polyuria is a characteristic symptom of the complication of diabetes[61]. In the present study, treatment with MK reduced the urine volume in DN animals. Glucose dependent pathways activated with DN include increased renal polyol formation, AGEs accumulation. These pathways ultimately lead to increased renal albumin permeability and extracellular matrix accumulation which in turn results in increasing UAE and glomerulosclerosis[62]. The results of the present study demonstrate that MEMK attenuate the development of UAE, prevent kidney structural injury and elevation of blood urea. This study also shows that MEMK reduced elevated blood urea level more effectively whereas preventive effect on UAE and GFR were comparable.Clearance tests mainly access the glomerular filteration rate (GFR). Measurement of GFR gives the general index for the assessment of the severity of renal damage[63]. Renal plasma clearance of fructose polysaccharide inulin is generally accepted as the standard references for the GFR by animal experiment[64]. In present study by the treatment with MEMK, inulin, urea and creatinine clearance was improved in DN animals. Changes in electrolytes, induced by diabetes are implicated in the complications of diabetes mellitus most commonly in the pathogenesis of nephropathy in humans[65]. In the present experiment, imbalance of electrolyte was elevated in DN rats. Treatment with MEMK balanced the concentration of electrolytes in order to prevent the complication in DN animals. HbA_1C can be used as an excellent marker of overall glycemic control. Since it is formed slowly and does not dissociate easily, it reflects the mean blood glucose level (MBG)[66]. Treatment with MEMK decrease hyperglycemia and therefore decreased the level of HbA_1C.

There have been reports that decreased GFR is associated with the formation of reactive oxygen intermediates[67]. The results of the present study show there was a significant correlation between renal dysfunction and renal oxidative stress. Chronic hyperglycemia, a well-recognized pathogenetic factor of long-term complications in diabetes mellitus, is reported to generate not only more ROS but also attenuates antioxidative mechanisms through glycation of the scavenging enzymes[67]. Lipid peroxidation of unsaturated fatty acids, one of the major reactions in vivo, has been proven to be an index of increased oxidative stress and the subsequent cytotoxicity[68]. STZ-induced diabetic nephropathic animals exhibiting significantly higher levels of lipid peroxides in renal homogenates suggests increased oxidative stress in diabetic kidneys. A marked improvement in renal function by MEMK in diabetic rats may involve its inhibitory effect on ROS, lipid peroxidation and subsequent formation of vasoactive mediators. The content of MDA is a good index of intensified oxidative stress in the tissues, showing enhanced peroxidation process[69.] In this study, the production of MDA was increased in DN rats. On treatment with MEMK reduced the level of MDA in DN rats. Significant diminution of SOD activities was repeated in the kidney of rats after 12 weeks of STZ induced diabetes. In present study the activity of SOD was markedly reduced in the DN group compared to normal control group. These changes have been ameliorated by MEMK which prove a renal protective role against oxidative damage in DN animals. GST belongs to a super family of multifunction isoenzymes playing a crucial role in the detoxifying mechanisms of drugs and xenobiotics by preventing the binding of reactive metabolites to cellular proteins and modulating the by-products of oxidative stress by catalyzing the conjugation of electrophilic moieties to

glutathione[70]. In present study GST levels was decreased in DN animals. Treatment with MEMK significantly increased GST levels in DN animals.

CONCLUSION

In present study, MEMK were attenuating the progression of DN and may be due to antioxidative properties. These results imply that MEMK could be used as an adjuvant therapy with a conventional hypoglycemic regimen to treat diabetic complications. Further studies are in progress in our research laboratory to isolate the active components from MEMK which are responsible for above study for attenuating the progression of DN.

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