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Research Article

Activity of Proline and its Analogs Isolated from *Murraya koenigii* Against Hyperglycemia, Oxidative Stress and Renal Insufficiency in Diabetic Nephropathy

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ABSTRACT

Proline is a major constituent of amino acids isolated from the *Murraya koenigii* leaves, which is used as a traditional medicine and have established efficacy against the treatment of life threatening chronic diabetes. Therefore based on modern medicine, isolated the bioactive components for their effects on hyperglycemia, hyperlipidemia and renal insufficiency produced by oxidative stress in diabetic nephropathy (DN) animals was undertaken to validate its traditional use. Bioassay guided fractionation and phytochemical investigation of the methanolic extract of the *M. koenigii* resulted in the isolation of Compound 1 (Trivial name: MF4) present in amino acid rich fraction and Compound 2 (Trivial name: Mk-F4). Compounds (1 and 2) were characterized by NMR, elemental analysis and GC-MS. MF4 and Mk-F4 were then investigated for their effects on hyperglycemia, hyperlipidemia and renal insufficiency produced by oxidative stress in DN animals. DN was induced in male adult albino rats by diabetogenic diet (for 7 days diet) and injecting streptozotocin (75 mg/kg b.wt., i.p.) on six weeks of administration. Treatment with Mk-F4 and MF4 at a dose of 50 mg/kg b.wt. p.o. for 40 days reduced the elevated levels of blood glucose, cholesterol, triglyceride, urea nitrogen, albuminuria, proteinuria and serum creatinine and increased the levels of high density lipoproteins and clearance of creatinine and urea in the DN animals. In addition, electrolytes sodium, calcium, chloride and magnesium levels in serum were markedly elevated and decreased the level of serum potassium in DN animals as compared with DN control groups. Furthermore, the oral administration of Mk-F4 and MF4 of *M. koenigii* significantly decreased the elevated level of malondialdehyde and increased radical scavenging enzyme activity. The renal pathological changes in treatment groups were ameliorated by marked improvement in chronic glomerulonephritis. These results indicate that *M. koenigii* extracts could be a very useful antioxidant for the prevention of renal insufficiency along with hyperlipidemic activity and provides a scientific rationale for its use as an antidiabetic agent.

Keywords: *M. Koenigi*, Streptozotocin, DN, Renal function, Oxidative stress

INTRODUCTION

Nephropathy is defined as the loss of functions of kidney associated with nephrotic syndrome, glomerulosclerosis, type IV renal tubular acidosis, persistant albuminuria, declining glomerular filtration rate. It's associated risk factors are high blood glucose, elevated cholesterol levels and proteinuria with the residual renal function[1]. In the diabetic state, multiple biochemical mechanisms, such as those involving growth factors and cytokines[2], activation of protein kinase C extracellular regulated protein kinase pathway[3,4], enhanced polyol pathway[5,6], and altered redox state and oxidative stress[7], have been proposed to be involved in the development of DN. Current approaches which modifies the progression of diabetic nephropathy include control of blood glucose, low protein diet, control of hypertension, hyperfiltration, usually through angiotensin converting enzyme inhibitor or angiotensin receptor blocking agents[8-10].

M. koenigii (Family: Rutaceae), commonly known as curry patta, a native of India and Sri Lanka, is a small tree with very pungent aromatic leaves. It is an important dietary source of amino acids and vitamins. Various parts of *M. koenigii* have been used in traditional or folk medicine for the treatment of rheumatism, traumatic injury and snake bite and it has been reported to have antioxidant, antidiabetic, antidysenteric, antimicrobial, anti-inflammatory, hepatoprotective and antihypercholesterolemic activities[11,12]. The green leaves are eaten raw for curing dysentery, and the infusion of the washed leaves stops vomiting[13]. However, in aqueous extracts or daily diet form very little is known about effect of leaves of *M. koenigii* in preventing or delaying the onset of diabetic complications[14]. Previously it has been reported that the methanolic extract of leaves of *M. koenigii* is effective in control of hyperglycemia and preventions of renal functions in DN animal[15]. However the active constituents of *M. koenigii* leaves have not yet been investigated intensively for its efficacy in attenuating diabetic nephropathy. Phytochemical investigation revealed the presence of amino acids in the leaves of *M.*

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koenigii[16]. The amino acids namely Proline and its analog N-Methyl Proline are reported to have antioxidant and a number of other pharmacological activities[17-21]. Researchers reported that the treatment with N-acetyl-seryl-aspartyl-lysyl-proline, prevents renal insufficiency and mesangial matrix expansion in diabetic db/db mice[22]. Recently reported that α-methyl-proline contains potential dual α4β1 integrin antagonist[23,24]. The hypotensive efficacy of (S)-1-[6-amino-2[[hydroxy(4-phenylbutyl) phosphinyl]oxy]-1-oxohexyl]-L-proline was also found to be effective in hypertensive dogs[26,27].

Thus the objective of the present study was to isolate the active phytoconstituents from the amino acids rich *M. koenigii* extract and to evaluate their efficacy in preventing renal insufficiency in STZ induced diabetic nephropathic animals which was selected on random as in the literature were taken for the screening of antidiabetics animal models[18]. This study provides the good evidence of a natural compound being useful as a novel therapeutic strategy for renal function.

MATERIAL AND METHODS

Drugs, chemicals and reagents

Streptozotocin was purchased from Sisco Research Laboratories, India. Standard antidiabetic drug glimipride was obtained from Aristo Pharmaceutical Pvt. Ltd., India. Analytical grade chemicals including various solvents from E. Merck India Ltd and Ranbaxy laboratories, India were used for the extraction, isolation and pharmacological studies.

Plant material

M. koenigii (L.) Spreng leaves were collected from the campus of Birla Institute of Technology, Mesra, Ranchi, and they were authenticated by K. Kanthigeyan, Scientitst 'C', Botanical Survey of India (BSI), Central National Herbarium, Howrah, with ref. no. CNH/103/2011/Tech-II/620 and the voucher specimen has been kept at the herbarium of Birla Institute of Technology, Mesra, Ranchi, Department of Pharmaceutical sciences for future reference.

Isolation and identification of active compounds

As reported previously[15,28], 500 gm of leaves of *M. koenigii* were ground and subjected to successive extraction with 15 times greater volume of petroleum ether, chloroform and methanol separately by hot soxhlation method[29]. After filtration of the extracts, MeoH extract was concentrated under reduced pressure on rotavapor (Buchi labortechnik AG, Flawil 1/Switzerland) to obtain a residue (6.0 % w/w) which was applied to a sephadex column (using slurry of silica gel 60-120 mesh, Merck and chloroform). Elution with n-butanol: acetic acid in 10, 25, 50 and 100% v/v, yielded four fractions represented by trivial name: MF1, MF2, MF3 and MF4. Further MF4 fraction was eluted with n-butanol: acetic acid: water (4:4:2 v/v/v) and were collected and subjected to repetitive preparative thin layer chromatography using silica gel G as stationary phase (20×20cm glass plates) and n-butanol:acetic acid:water (4:4:2 v/v/v) as mobile phase. It led to the isolation of single pure compound which is denoted by trivial name Mk-F4. On the other hand MF4 fraction (yield: 4.2% w/w) and Mk-F4 (yield: 2.5% w/w), both were identified and characterized by CHNO (Elementar, Vario EL III), GC-MS (GC-MS-QP-2010-Plus1, Shimdzu), ^{1}H NMR and ^{13}C NMR spectra (Bruker DRX 400 NMR spectrometers with MeOD) with those of authentic specimens.

Acute toxicity study

Acute toxicity study was carried out for fraction MF4 following OECD 423 guidelines[30]. Drug 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=3). 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.

Animal experiments

Davis *et al* reported the different animal models for the induction of diabetic nephropathy[31]. It was seen that a low dose of STZ (55 mg/kg i.p.) did not develop sufficient diabetes to cause significant renal injury. The STZ dose was increased to 200 mg/kg i.p. developed nephropathy after 24 h which resulted from hyperglycaemia-induced injury super imposed on acute renal STZ cytotoxicity. Rakieten *et al* reported the diabetogenic effect and cytotoxicity to beta-cells of pancreas after 24 h with the administration of streptozotocin at a dose of 200 mg/kg b.wt., i.p[32]. Therefore in present study in reference of above mentioned method, diabetic nephropathy animal (adult albino rat) model (trial and error) was successfully established based on limit dose of STZ adminstration and diabetogenic diet which was selected on random and limit basis as in the literature were taken for the screening of diabetic nephropathy.

Method: The guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of the Govt. of India were followed and prior permission and clearance were granted from the Institutional Animal Ethics Committee (BIT/PH/IAEC/32/2011, Dated: 10/12/2011) for conducting the animal experiments. Inbred male adult albino rats were housed in standard polypropylene cages and were controlled automatically under room temperature(22+2 $^{\circ}$C) and humidity (55+5%) with 12:12 hour light and dark cycle. The animals were fed either regular diet for good health or diabetogenic diet that induced diabetic phenotype in animals depending on the group[33]. 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 acclimatization, overnight fasted animals (given access to drinking water, for 16 hr) were injected bolus of 75 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 and animals having fasting blood sugar (FBS) above 200 mg/dl was considered to be diabetic[33]. At a gap of 3 weeks, the animals were estimated for the induction of diabetic nephropathy using different renal function test i.e. blood urea nitrogen (BUN), creatinine, albuminuria (UAE) and total protein including body weight[34-35]. Nephropathy was noted in animals on 6th week after the administration of STZ (75 mg/kg b.wt, i.p, once) as assessed in terms of above mentioned renal function test. Treatment was given for 40 days which started on the 6th week after the induction of diabetic nephropathy. The biochemical and pharmacological studies were carried out on 0th, 10th, 20th, 30th and 40th day of the experiment.

Treatment Protocol

After the induction of diabetic nephropathy was found to be produced in those animals on the 6th week of induction. Treatment was from the 6th week which was taken as the 1st week of induction of nephropathy and 0th day of treatment. The animals were divided into five groups each containing six animals.

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: Diabetic nephropathic animals received saline daily, p.o., and served as a diabetic nephropathic control (DN).

Group III. Diabetic nephropathic animals received glimepiride at a dose of 2mg/kg, p.o., and served as standard group (TS).

Group IV: Diabetic nephropathic animals received active fraction MF4 at a dose of 50 mg/kg, p.o, and served as first treatment group (MF4).

Group V: Diabetic nephropathic animals received isolated compound Mk-F4 at a dose of 50 mg/kg, p.o, and served as second treatment group (Mk-F4).

Group IV and V DN animals were compared with Group III DN animals. It has been evident from literature review that glimepiride showed the reduction in renal marker and oxidative stress in ischemia/reperfusion induced renal marker in diabetic rats[37]. So glimepiride used as standard drug in present study where presence of active constituents in leaves of *M. koenigii* was used for comparative study to see its efficacy in attenuating the DN in induced DN animals.

Sample collection

During the treatment period, fasting blood sample was collected from the retro-orbital region of the inner canthus of the eye under light ether anaesthesia using capillary tubes (Micro Hematocrit capillaries, Mucaps). Serum was separated in a cold centrifuge (Remi, C-24 BL) at 2000 rpm for 10 minute and the urine sample of animals were collected by keeping the animals in metabolic cages for 24 hours with proper access to drinking water and food.

Serum and urine biochemical analysis

Serum was taken from all the groups and analyzed for fasting blood sugar by glucose oxidase-peroxidase method[37], lipid profile including total cholesterol (TC), high density lipoprotein (HDL) and triglyceride (TG) levels based on Chod-PAP method[38]. The renal function test were determined by different biochemical parameters such as BUN based on modified berthelot method[39], serum creatinine (SCr) based on Jaffe's kinetic method[40], UAE using BCG method[41] and proteinuria (TP) by Lowry method[42], creatinine clearance (CrC_l) and urea clearance (UrC_l) were determined for measuring glomerular filteration rate[43]. Serum electrolytes concentrations (sodium, potassium, chloride, calcium and magnesium) were assayed respectively[44-48].

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[49]. The kits used for determination of the above parameters were obtained from Span diagnostics Ltd. Sachin, Surat, India.

Kidney hypertrophy, oxidative stress, renal enzymes status and its histopathological studies

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 (RH) was expressed as the ratio of the weight of the two kidney to total body weight[50]. Then one kidney was kept at -20^{0}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 40^{o}C. The resulting supernatant was used for enzyme assays by malondialdehyde (MDA)[51], superoxide dismutase (SOD)[52], catalase[53] and glutathione s-transferase activities (GST)[53]. The other kidney was fixed in 10% formal saline for renal histopathological examination[54].

Statistical analysis

Statistical analysis was performed using graph pad prism software trial 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.

RESULTS

Characterization of the isolated compounds from M. koenigii extract

Phytochemical analysis (Millon's and Ninhydrin test) of the Mk-F4 and MF4 confirmed its amino acid nature.

Fraction MF4: Presence of components in fraction were identified and characterized as: ^{13}C-NMR (100 M HZ, MeOD): 12.83, 18.62, 20.07, 22.76, 28.73, 29.37, 34.40, 39.97, 47.10, 47.27, 47.44, 47.61, 47.78, 47.89, 47.95, 48.06, 48.12, 48.24, 55.85, 61.27, 70.75, 72.04, 72.74, 72.91, 75.10. GC-MS: *RT* 10.589 (95.99 %), MW: 115.02, corresponding to proline skeleton. This compound exhibited comparable spectroscopic data (NMR) to published values[55,56]. Combination of all spectral data led to the conclusion that the fraction containing MF4 or compound (1) is Proline. *Mk-F4:* GC-MS: *RT* 9.818, MW: 129; formula - $C_6H_{11}NO_2$, CHNO analysis: compound contains 55.69 % of C, 8.52 % of H, 10.65 % of N and 24.61 % of O. ^{1} H-NMR (400 M HZ, MeOD): δ 1.6 (m, 2H, H-4), 1.9 (m, 2H, H-3), 2.2 (s, 3H, H-7), 2.3 (m, 2H, H-5), 3.1 (s, 1H, H-2, J = 7.0). ^{13}C-NMR (100 M HZ, MeoD): δ 19.46 (C-4), 22.75 (C-3), 39.97 (C-2), 55.85 (C-

5), 70.77 (C-7), 171.76 (C-6). Spectrum of this compound resembled data published in previous studies[56]. Combination of all spectral data led to the conclusion that Mk-F4 is the single pure compound (2) and is N-Methyl Proline. These two structural formulae isolated from *M. koenigii* extract are given in Figure 1.

Acute toxicity study

Based on OECD 423 guidelines test animals did not exhibit any visible change and survived beyond recommended duration of observation with 300 mg/kg b wt. of MF4. The next dose was 2000mg/kg, in which two of animals survived due to the wide gap in dosing[57]. Drugs were safe up to 300 mg/kg. Hence the doses 50 mg/kg of MF4 and Mk-F4 were selected based on limit dose for pharmacological study.

Effect of Mk-F4 and MF4 on blood glucose, and lipid profile in DN animals

Table 2 illustrates the effect of Mk-F4 and MF4 on blood glucose and lipid profile in the DN animals. Results showed that, Mk-F4 ($P<0.01$) and MF4 ($P<0.05$) exhibited significant reduction in fasting blood sugar in DN animals. Mk-F4 and MF4 caused significant ($p<0.05$) reduction in the cholesterol, triglyceride and significant ($P<0.01$) increase in HDL levels in the DN animals after treatment.

Effect of Mk-F4 and MF4 on renal function tests in DN animals

Table 2 shows the BUN and SCr levels. Mk-F4 showed significant reduction ($P<0.05$) and MF4 insignificant reduction in the BUN in DN animals. Treatment with Mk-F4 and MF4 reduced the serum creatinine levels significantly ($P<0.05$) on 40th day as compared to DN group. Table 3 shows the UAE, TP, CrCl and UrCl levels. Mk-F4 and MF4 caused significant ($P<0.05$) reduction in UAE, TP and significant ($P<0.05$) increase in CrCl and UrCl levels in the DN animals after treatment.

Effect of Mk-F4 and MF4 on serum electrolytes concentration, GHb and RH levels in DN animals

Table 4 illustrates the effect of Mk-F4 and MF4 on serum electrolytes level in the normal and DN animals. Mk-F4 and MF4 caused significant ($P<0.05$) increase in the serum sodium, chloride, calcium and magnesium and significant ($P<0.05$) decrease in potassium levels in the diabetic rats after 40th day of treatment. Table 5 showed a significant decrease ($p<0.05$) in the GHb in the DN animals as compared with the NC group. And Table 4 showed a significant decrease ($P<0.05$) in the RH in the DN animals after treatment.

Effect of Mk-F4 and MF4 on renal antioxidant status and histopathological study of DN animals Table 4 showed a significant decrease ($P<0.05$) in the MDA and significant increase in SOD, GST and catalase levels in the DN animals as compared with the NC group after 40th day of treatment with Mk-F4 and MF4. 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, enlarged glomeruli and the subcapsular space was small. On 40th day of treatment with Mk-F4 and MF4, normal glomerulus
was seen as compared to the DN group.

DISCUSSIONS

In the present study, Proline (MF4) and its analog N-Methyl Proline (Mk-F4) isolated from the leaves of *M. koenigii* have been used in traditional medicine[18], specifically for the treatment of hyperglycaemia, hyperlipidemia and renal insufficiency with oxidative stress in STZ induced DN animals. Free radicals are highly reactive and injure lipids and proteins, thus resulting in structural and functional abnormalities. Now a days a few of the antioxidants have been studied which show protection against renal damage[58]. In the present study, Mk-F4 and MF4 has been used for the treatment of DN animals and results showed improvement in control of glycemic condition. Hyperglycemia in diabetic patients is associated with alteration of glucose and lipid metabolism and modification in liver enzyme level. Liver is an important insulin sensitive tissue which regulates glucose and lipid homeostasis under the influence of insulin. In diabetes due to lack of insulin all of these processes gets affected[59]. In present study DN animals develop hyperlipidemia, as reflected by elevated levels of total cholesterol and triglycerides. This is consistent with previous reports in diabetic animals that demonstrate a strong correlation between lipid profile and progression of DN[60]. In the present study, it has been concluded that Mk-F4 and MF4 showed the decrease in FBG and cholesterol levels in diabetic nephropathic animals as is also supported by the literature of *M. koenigii* having antidiabetic and hypolipidemic activity[18,61]. The level of cholesterol and triglyceride increases where as HDL level decreases in DN animals. These findings have been well justified by various reports in the literature stating that increased cholesterol concentration is found in nephrotic syndrome, chronic glomerulonephritis, neprosis and diabetes mellitus[62]. These defects seem to be due in part to increased synthesis of lipoporotein in the liver, abnormal transport of circulating lipid particles and decreased catabolism. HDL is also lost in the urine when severe proteinuria occurs. Lipiduria follows hyperlipidemia because of lipoprotein leakage across the glomerular capillary wall. The lipid appears in the urine either as free fat or as oval fat bodies representing lipoproteins absorbed by tubular epithelial cells. Cholesterol is synthesised in many tissues from acetyl CoA and is usually eliminated from the body in the bile as cholesterol or bile salts[63]. Administration of Mk-F4 and MF4 restores the level of cholesterol and triglyceride near to the normal value. This also caused increase in the level of HDL cholesterol in DN animals.Urea is the end product of protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels of urea are found in renal diseases[64]. During the progression of diabetes, the elevation of the serum BUN and Cr levels are due to the

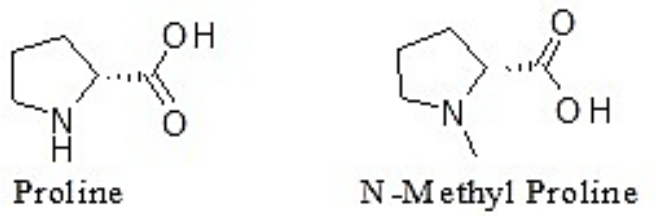

Figure 1: Isolated compounds from the leaves of *M.Koenigii*

Table 1: Changes of the blood parameters in DN animals receiving 40 days of MF4 and Mk-F4 treatment

Groups	FBG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	STG (mg/dl)	BUN (mg/dl)	SCr (mg/dl)
NC	83.02 ± 10.4	163.82 ± 3.6	47.16 ± 1.4	84.34 ± 4.2	26.34 ± 2.4	0.31 ± 0.01
DN	401.38 ± 19.8[a]	259.14 ± 5.9[a]	33.97 ± 1.2[a]	180.57 ± 3.2[a]	42.81 ± 3.6[a]	0.58 ± 0.01[a]
TS	210.34 ± 12.7[b]	158.35 ± 4.8[b]	52.56 ± 2.1[b]	93.13 ± 4.5[b]	21.63 ± 4.2[c]	0.33 ± 0.04[b]
MF4	298.31 ± 9.6[c]	210.24 ± 9.3[c]	43.25 ± 0.6[c]	109.15 ± 7.2[c]	30.02 ± 3.2[c]	0.38 ± 0.02[c]
Mk-F4	271.60 ± 15.5[c]	198.25 ± 6.1[c]	48.01 ± 1.3[c]	95.26 ± 1.2[c]	22.37 ± 2.7[c]	0.34 ± 0.01[c]

Values (mean ± S.E.M.) were obtained for each group of 6 animals.
aP < 0.001 compared to the values of NC at the corresponding time, respectively.
bP < 0.01 and cP < 0.05 compared to the values of DN at the corresponding time, respectively

Table 2: Changes of the urine parameters in DN animals receiving 40 days of MF4 and Mk-F4 treatment

Groups	UAE (µg/24 hr)	TP (g/100 ml)	CrC_l (ml/24hr)	UrC_l (ml/24hr)
NC	412.3 ± 18.9	0.233 ± 0.02	0.72 ± 0.01	0.69 ± 0.03
DN	1352.6 ± 77.5[a]	1.895 ± 0.08[a]	0.31 ± 0.02[a]	0.34 ± 0.01[a]
TS	618.3 ± 36.8[b]	0.257 ± 0.06[c]	0.70 ± 0.04[b]	0.64 ± 0.08[b]
MF4	682.2 ± 43.7[c]	0.369 ± 0.11[c]	0.61 ± 0.01[c]	0.49 ± 0.03[c]
Mk-F4	637.0 ± 38.8[b]	0.314 ± 0.06[c]	0.64 ± 0.01[c]	0.57 ± 0.04[c]

Values (mean ± S.E.M.) were obtained for each group of 6 animals.
aP < 0.001 compared to the values of NC at the corresponding time, respectively.
bP < 0.01 and cP < 0.05 compared to the values of DN at the corresponding time, respectively

accumulation of extracellular matrix in the glomeruli and interstitium, which also leads to renal fibrosis known as DN[65]. CrCl level, the most renal function index, was significantly improved in the treatment groups with a lower level of BUN and Cr, which indicates the protective effect of renal function 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[66]. Urine albumin is now the preferred measure of urine protein in patients with diabetes. Quantification of the UAE rate is useful for identifying diabetic patients at risk for developing DN[67]. In present study UAE levels was improved in the treatment groups and prevent kidney structural injury. Changes in electrolytes, induced by diabetes are implicated in the complications of diabetes mellitus most commonly in the pathogenesis of nephropathy in humans[68]. In the present experiment, imbalance of electrolyte was elevated in DN animals. Treatment groups have 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)[69]. Treatment with Mk-F4 and MF4 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[70]. The results of the present study showed 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[71]. 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[72]. 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 Mk-F4 and MF4 in diabetic animals 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[73]. In this study, the production of MDA was increased in DN animals. Treatment groups reduced the level of MDA in DN animals. Significant diminution of SOD activities was repeated in the kidney of animals after 40 days of treatment of STZ induced animals. 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 Mk-F4 and MF4 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

Table 3: Effect of 40 days treatment of MF4 and Mk-F4 on serum electrolyte concentrations, renal hypertrophy and renal antioxidant status of DN animals

Parameters	NC	DN	TS	MF4	Mk-F4
Sodium (meq/L)	162.11 ± 0.09	148.53 ± 0.32[a]	156.85 ± 0.26[b]	153.14 ± 0.31[c]	156.09 ± 0.02[c]
Potassium (meq/L)	5.22 ± 0.08	6.42 ± 0.01[a]	5.16 ± 0.05[b]	5.29 ± 0.02[c]	5.23 ± 0.05[c]
Chloride (meq/L)	96.13 ± 0.23	81.12 ± 0.15[a]	95.13 ± 0.21[b]	90.23 ± 0.10[c]	93.33 ± 0.12[c]
Calcium (meq/L)	8.85 ± 0.10	7.57 ± 0.06[a]	9.12 ± 0.06[b]	8.98 ± 0.03[c]	9.12 ± 0.04[c]
Magnesium (meq/L)	5.01 ± 0.08	1.90 ± 0.05[a]	4.35 ± 0.20[b]	3.23 ± 0.09[c]	3.98 ± 0.06[c]
RH (gm)	0.004 ± 0.001	0.009 ± 0.002[c]	0.004 ± 0.001[c]	0.005 ± 0.001[c]	0.005 ± 0.02[c]
MDA(µM/mg of renal)	1.18 ± 0.83	3.98 ± 0.20[c]	1.42 ± 0.09[c]	1.58 ± 0.02[c]	1.58 ± 0.01[c]
SOD(EU/mg protein)	51.30 ± 0.36	31.20 ± 0.12[a]	50.21 ± 0.06[b]	48.20 ± 0.01[c]	48.81 ± 0.03[c]
Catalase (µM/min/mg)	31.23 ± 0.32	16.21 ± 0.14[a]	30.21 ± 0.10[b]	27.31 ± 0.07[c]	27.94 ± 0.04[c]
GST(nM/mg protein)	0.20 ± 0.04	0.09 ± 0.01[a]	0.21 ± 0.02[b]	0.15 ± 0.02[c]	0.20 ± 0.03[c]

Values (mean ± S.E.M.) were obtained for each group of 6 animals.
[a]*P < 0.001* compared to the values of NC at the corresponding time, respectively.
[b]*P < 0.01* and [c]*P < 0.05* compared to the values of DN at the corresponding time, respectively.

Table 4: Effect of 40 days treatment of MF4 and Mk-F4 on glycosylated hemoglobin levels in DN animals

Parameter	NC	DN	TS	MF4	Mk-F4
GH (%)	6.1	17.1[a]	9.3[c]	10.6[c]	10.1[c]

Values (mean ± S.E.M.) were obtained for each group of 6 animals.
[a]*P < 0.001* compared to the values of NC at the corresponding time, respectively.
[b]*P < 0.01* and cP < 0.05 compared to the values of DN at the corresponding time, respectively.

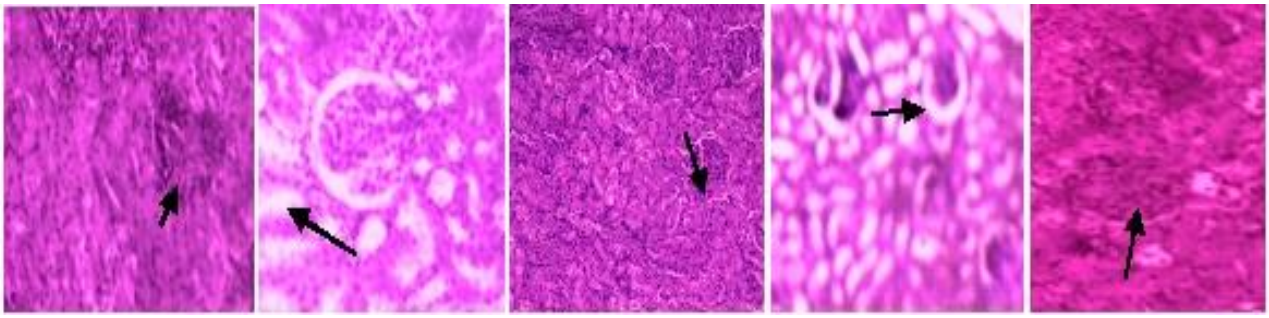

Figure 2: Photomicrographs of the glomeruli of diabetic nephropathic control group, DN; normal control, NC; standard group, TS; treatment groups, MF4 and Mk-F4

binding of reactive metabolites to cellular proteins and modulating the by-products of oxidative stress by catalyzing the conjugation of electrophilic moieties to glutathion[74]. In present study GST levels was decreased in DN animals. Treatment groups showed significantly increased GST levels in DN animals. In the DN animals, there was histological evidence of advanced renal damage: nodular glomerulosclerosis, hyaline arteriolosclerosis, enlargement of glomeruli, capillary tuft and of subcapsular urinary space. On 40th day of treatment with Mk-F4 and MF4, normal glomerulus was seen as compared to the DN group. In the present study, it was demonstrated that renal dysfunction is mediated by increases of blood glucose, lipid, and proteinuria with oxidative stress in DN animals. The functional components of foods plays a vital role in the maintenance of health and prevention of diseases[75]. *M. koenigii* is extensively eaten as food flavouring agent in India and Sri Lanka. Thus the methods are now being developed to make good use as traditional medicine of these functional food components. But the functions of such dietary medicine in body is not well known and limited, and further study of such medicine for curing diabetes and its associated complications is urgently needed. The isolated active components from *M. koenigii* which may shows its action as daily traditional medicine. In the present study, the administration of Mk-F4 and MF4 imply its potential efficacy in preventing the renal insufficiency by inhibiting the hyperglycaemia in STZ induced DN animals and may be due to antioxidative properties. These findings suggest that may be examples of this kind of bioactive natural products could be used as an adjuvant therapy with a conventional hypoglycemic regimen to treat diabetic complications and kidney diseases. In the present study, the dose of extracts and isolated compounds taken for the study is selected on random and limit basis as in the literature were taken for the screening of antidiabetics. However further preclinical studies are required to completely evaluate the exact mechanism of action of Proline and N-Methyl Proline, so that they can be used as a lead molecule for the synthesis of other potent antidiabetic drugs and novel drugs for the treatment of life threatening diseases i.e. long term complication of diabetes like nephropathy, retinopathy and neuropathy.

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