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 function1. In the diabetic state, multiple biochemical mechanisms, such as those involving growth factors and cytokines2, activation of protein kinase C extracellular regulated protein kinase pathway3,4, enhanced polyol pathway5,6, and altered redox state and oxidative stress7, 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 agents8,10. *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, antidiysenteric, antimicrobial, anti-inflammatory, hepatoprotective and antihypercholesterolemic activities11,12. The green leaves are eaten raw for curing dysentery, and the infusion of the washed leaves stops vomiting13. 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 complications14. 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 animal15. 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.
koenigii\textsuperscript{16}. The amino acids namely Proline and its analog N-Methyl Proline are reported to have antioxidant and a number of other pharmacological activities\textsuperscript{17-21}. Researchers reported that the treatment with N-acetyl-L-aspartyl-L-lysyl-proline, prevents renal insufficiency and mesangial matrix expansion in diabetic db/db mice\textsuperscript{22}. Recently reported that α-methyl-proline contains potential dual α4β1 integrin antagonist\textsuperscript{23,24}. The hypotensive efficacy of (S)-[6-amino-2[(hydroxy(4-phenylbutyl) phosphinyl]oxy]-L-proline was also found to be effective in hypertensive dogs\textsuperscript{25,26}. 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 antiadibetics animal models\textsuperscript{27}. 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 anti diabetic 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, Scientist ‘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\textsuperscript{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\textsuperscript{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 (20x20cm 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, Shimadzu), \textsuperscript{1}H NMR and \textsuperscript{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\textsuperscript{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\textsuperscript{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.w., i.p\textsuperscript{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 administration 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 (B/IT/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 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\textsuperscript{33}. Regular diet consisting of a mixture of ground whole wheat, normal pellet (Foster Biotech, India, Ldt. 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 diabetic33. 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 weight34-35. 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 rats36. 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 minutes 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 method57, lipid profile including total cholesterol (TC), high density lipoprotein (HDL) and triglyceride (TG) levels based on Chod-PAP method58. The renal function test were determined by different biochemical parameters such as BUN based on modified berthelot method39, serum creatinine (Scr) based on Jaffé’s kinetic method40, UAE using BCG method41 and proteiniuria (TP) by Lowry method42, creatinine clearance (CrCl) and urea clearance (UrCl) were determined for measuring glomerular filtration rate43. Serum electrolytes concentrations (sodium, potassium, chloride, calcium and magnesium) were assayed respectively44-46. 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 method49. 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 weight50. Then one kidney was 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 40°C. The resulting supernatant was used for enzyme assays by malondialdehyde (MDA)51, superoxide dismutase (SOD)52, catalase53 and glutathione s-transferase activities (GST)54. The other kidney was fixed in 10% formal saline for renal histopathological examination55.

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: 1H-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 values55-56. 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 - C₂H₉N₂O₂, CHNO analysis: compound contains 55.69 % of C, 8.52 % of H, 10.65 % of N and 24.61 % of O. 1H-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). 13C-NMR (100 M Hz, MeOδ): δ 19.46 (C-4), 22.75 (C-3), 39.97 (C-2), 55.85 (C-
Urea is the end product of protein metabolism. It is synthesized in the liver and excreted in the urine. Increased levels of urea are found in renal diseases where it is excreted. Increased levels of urea are due to the breakdown of amino acids. It is transported by the blood to the kidneys from the ammonia produced by the catabolism of amino acids. It is transported in the urine either as free fat or as oval fat bodies.
accumulation of extracellular matrix in the glomeruli and interstitium, which also leads to renal fibrosis known as DN. 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 polyl 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. 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. 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. 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. HbA1C 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). Treatment with Mk-F4 and MF4 decrease hyperglycemia and therefore decreased the level of HbA1C. There have been reports that decreased GFR is associated with the formation of reactive oxygen intermediates. 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. 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. 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 antioxidative mechanisms through glycation of the scavenging enzymes. 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. 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 increased oxidative stress in the tissues, showing enhanced peroxidation process. 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
binding of reactive metabolites to cellular proteins and modulating the by-products of oxidative stress by catalyzing the conjugation of electrophilic moieties to glutathione. 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. 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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