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Renoprotective Effects of Reconstructed Composition of *Trigonella foenum-graecum* L. Seeds in Animal Model of Diabetic Nephropathy with and without Renal Ischemia Reperfusion in Rats

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Abstract: Diabetic nephropathy is the kidney complication of diabetes mellitus and leading to end stage renal disease. In the past, reconstructed antidiabetic combination from *Trigonella foenum-graecum* (fenugreek) seeds (IND01) containing 4-hydroxyisoleucine (40%), trigonelline (30%) and galactomannan (30%) showed excellent antihyperglycemic activity. The objective of the present study was to evaluate the renoprotective efficacy of IND01 in animal model of diabetes with and without ischemia reperfusion injury. The diabetes was induced by alloxan in dose of 160 mg kg⁻¹, intraperitoneally (early nephropathy model, without ischemia reperfusion) or 70 mg kg⁻¹, i.v. (model with ischemia reperfusion). In both models, effects of oral treatment of IND01 (50, 100 and 200 mg kg⁻¹, daily once for 30 days) were observed on biochemical parameters (creatinine clearance) and urine (blood urea nitrogen). On day 30, rats were sacrificed and histology was performed on isolated kidneys. Alloxan administration with or without ischemia reperfusion showed symptoms of severe nephropathy (decreased creatinine clearance, increased BUN, presence of glomerular matrix formation, tubular necrosis, interstitial inflammation and fibrosis). The daily oral administration of IND01 (50-200 mg kg⁻¹) showed potent and mild renoprotective effects on biochemical parameters against diabetic rats without ischemia (early nephropathy) and with ischemia model respectively. IND01 showed moderate protection from histological abnormalities in kidney of alloxan-induced rats without ischemia reperfusion injury (early nephropathy model). However, such protection was not offered by IND01 in alloxan induced rats with ischemia reperfusion injury. In conclusion, IND01 showed renoprotection in animal model of early nephropathy probably by effective glycemic control.

Key words: *Trigonella foenum-graecum* (fenugreek) seeds, diabetic nephropathy, creatinine clearance, diabetes complications

INTRODUCTION

Diabetic nephropathy (DN) is the kidney disease that occurs as a result of diabetes mellitus (DM). DN is the single most common disorder leading to renal failure (Sampanis, 2008). Between 20 and 40% of patients with diabetes, eventually develop DN, which is the leading cause of end-stage renal disease (ESRD) and needs dialysis or kidney transplant (Sampanis, 2008). Both clinical and experimental data suggest that hyperglycemia increases the risk of acute renal failure (Wald *et al.*, 1990). Conversely, ischaemia-reperfusion (I/R) combined with hyperglycemia could also be important in the development of diabetic nephropathy (Melin *et al.*, 2003). After many years of DM, the delicate filtering system in

the kidney gets destroyed, becoming leaky to large blood proteins such as albumin that are lost in urine. Its incidence and prevalence has increased to alarming proportion in the past decade despite recent therapeutic advances.

In patients with longer lasting DM, particularly in the so-called decompensated DM, the occurrence of specific and non-specific chronic complications are common (Brilla, 1997). DN is a result of interaction of metabolic and haemodynamic factors. Glucose-dependant pathways are activated within the diabetic kidney. The causes for this activation include increased oxidative stress, renal polyol formation, advanced glycated end-products (AGEs) accumulation and presclerotic cytokines, such as transforming growth factor- β 1 (TGF- β 1). These pathways

eventually lead to increase in renal albumin permeability and extra cellular matrix build-up, which results in increasing proteinuria, glomerulosclerosis and tubulointerstitial fibrosis.

DN patients need to maintain the vital functions and improve the quality of life. This objective is only possible by reducing the need of dialysis and improving their renal functions to normal levels. Currently, Angiotensin Converting Enzyme (ACE) inhibitors and angiotensin-II (AT-II) receptor blockers are used to treat DN. Although, these drugs are useful, they need to be monitored as they may have detrimental effects in some people. Novel targets, which are linked to glucose dependent pathways, are the major focus of new therapies directed against diabetes induced renal damage. It is likely that resolution of DN will require synergistic therapies to target multiple mediators of this disease (Bachar and Lichtstein, 1993). Hence, the nephropathic population of patients is in dire need for alternate medication.

Fenugreek (*Trigonella foenum-graecum* L. Family: Leguminosae), a spice rich in dietary fibres has traditional history of medicinal use in the management of diabetes. Anecdotal report suggest a 3000 year old history of medicinal use for fenugreek in Egypt, Southern Europe, India, Asia and northern Africa (WHO, 2007). In recent decades, several health beneficial physiological attributes of fenugreek seeds have been reported in animal studies as well as human trials (Ulbricht *et al.*, 2007; Kang *et al.*, 2008). *Trigonella foenum-graecum* seeds have previously been shown to have hypoglycemic and hypocholesterolemic effects on type 1 and type 2 DM patients (Sharma *et al.*, 1990; Gupta *et al.*, 2001; Yadav *et al.*, 2008) and experimental diabetic animals (Jelodar *et al.*, 2005; Abdelatif *et al.*, 2012). *Trigonella foenum-graecum* seed extract showed improvement in diabetic rats in a dose-dependent manner (Xue *et al.*, 2007). The parameters like kidney to body weight ratio, blood glucose, glycated haemoglobin (HbA1c), triglycerides, total cholesterol and higher-density-lipoprotein-cholesterol lipid peroxidation and antioxidant status and hemorheological properties were improved in alloxan-induced diabetic rats (Xue *et al.*, 2007).

Recently, efficacy of fenugreek oil in to ameliorate DM and improvement in renal toxicity is reported (Hamden *et al.*, 2010). Oral administration of fenugreek seed powder for 3 weeks in alloxan-induced diabetic rats stabilized glucose homeostasis and free radical metabolism in kidney (Thakran *et al.*, 2003). Further, protective effects of fenugreek seed powder on kidney histology of toxicant-induced kidneys are reported (Thakran *et al.*, 2004; Sushma and Devasena, 2010). However, the exact composition responsible for beneficial effect on DM induced renal complications is yet challenge.

Fenugreek seed mainly contain 4-hydroxyisoleucine (4-HI), trigonelline, galactomannan with flavonoids, carotinoids, coumarins, proteins, saponins and lipids (Al-Habori *et al.*, 2001; Basch *et al.*, 2003). Individual constituents of fenugreek seeds like 4-HI (Sauvaire *et al.*, 1998; Broca *et al.*, 2000; Broca *et al.*, 2004; Narender *et al.*, 2006; Singh *et al.*, 2010), trigonelline (Mishkinsky *et al.*, 1967; Shah *et al.*, 2006a), galactomannans (Hannan *et al.*, 2003; Hannan *et al.*, 2007; Kamaljit *et al.*, 2011) demonstrated potent antidiabetic effect in animals. 4-HI has been found to be a major free amino acid in the seeds (Sauvaire *et al.*, 1976). Trigonelline is a major alkaloid constituent of the fenugreek seeds (Mishkinsky *et al.*, 1967). Galactomannans are polysaccharides consisting of mannose backbone with galactose side groups. The defatted fenugreek seed contains about 40% of 4-HI, 30% of trigonelline and 30% of galactomannan. In the past, we have reported antihyperglycemic activity of reconstructed composition of fenugreek seeds, IND01, which contains 4-HI (40%), trigonelline (30%) and galactomannan (30%) (Shah *et al.*, 2006b). IND01 also reported to have synergistic antihyperglycemic interaction with synthetic antihyperglycemic molecules like pioglitazone and glyburide (Shitole *et al.*, 2009). However, effects of IND01 on renal complications of diabetes are yet unknown. The aim of present work was to evaluate effects of long-term treatment of IND01 in kidneys of diabetic rats with or without renal I/R.

MATERIALS AND METHODS

Drugs and chemicals: The preparation of IND01 involve the extraction, isolation and characterization of 4-HI, trigonelline and low molecular weight galactomannan and was carried out as per reported methods (Shah *et al.*, 2006a,b; Shah *et al.*, 2009). These isolated phytochemicals were mixed in proportion of 40:30:30, respectively to get reconstructed composition product, IND01. The test solution of IND01 was freshly prepared in by dissolving in distilled water in concentration of 100 mg mL⁻¹ and was administered to animal based on body weight. Alloxan monohydrate (Sigma-Aldrich, USA.), pioglitazone (Ajantha Pharma Pvt. Ltd., India) and insulin: (Mixtard 30 HM, 40 IU mL⁻¹ Novo Nordisk) and anaesthetic ether (TKM Pharma, Hyderabad, India) were purchased from respective vendors. Biochemical kits for estimation of creatinine (Jaffe method), urea (Enzymatic method) and glucose (Accurex Biomedical Pvt. Ltd., Mumbai, India) were purchased respective vendors.

Animals: Male Wistar rats (150-200 g) were purchased from National Toxicology Centre, Pune. During the experiment, rats were housed at standard housing condition like temperature of 25±1°C, relative humidity of

45-55% and 12 h. light: 12 h dark cycle. Research protocol was approved by Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune. The rats had free access to food pellets (Amrut Laboratory, Navmaharashtra Chakan Oil Mills Ltd., Sangli, India) and tap water *ad libitum* during the experiments.

Early nephropathy in diabetic rats (Mishra *et al.*, 2010):

All rats except those of Group I, i.e., normal or Non-diabetic, were treated with alloxan (160 mg kg⁻¹, i.p.) to induce hyperglycemia and diabetes. Serum Glucose (SG) levels of rats were determined after 48 h of injection (day 0 of study) using Accurex glucose estimation kits. Rats with serum glucose above 300 mg dL⁻¹ were selected. The selected diabetic rats were divided into group II to VI of 6 rats each. Rats of group II and III were orally gavaged with distilled water (1 mL kg⁻¹) and standard antidiabetic agent, pioglitazone (10 mg kg⁻¹) once a day for 30 days, respectively. Rats from group IV, V and VI were orally gavaged with IND01 at dose of 50, 100 and 200 mg kg⁻¹, respectively once a day for 30 days. The biochemical parameters of all rats were measured on 0, 15 and 30th day and histological studies were done on kidney samples after sacrifice on 30th day.

Renal I/R injury in diabetic rats (Melin *et al.*, 2002): All rats except those of Group A, i.e., normal or Non-diabetic, were treated with alloxan (70 mg kg⁻¹, i.v.) to make them diabetic. Serum Glucose (SG) levels of rats were determined after 48 h of injection (day 0 of study) using Accurex glucose estimation kits. Rats with SG levels above 300 mg dL⁻¹ were selected for this study. Selected rats were randomly divided into groups of 6 rats (Group B to G). Seven days before induction of renal I/R, Group A, B and C had received once daily treatment of vehicle (distilled water) for 7 days before I/R surgery. Rats in Group D were pretreated with insulin (8 IU, s.c.) daily for 7 days. Rats from group E, F, G were pretreated with IND01 (50, 100 and 200 mg kg⁻¹, p.o. daily once), respectively for 7 days.

After completion of 7 days of pretreatment (Day 0 of study), renal I/R was induced in the rats of group C to G. Group B rats, called as sham control, had small cut in skin but no I/R induction. Group A rats (Normal) neither had I/R or cut to the skin. The rats from group C, D, E, F and G were anaesthetized by intraperitoneal (i.p.) injection of thiopental sodium in a dose of 50 mg kg⁻¹. During the operation the animals were placed on a servo-controlled heating pad keeping the body temperature at 37.5°C. A left flank incision was made and the left renal artery was located and dissected free from its surrounding structures. After a recovery period of 10 min, a small midline incision was made to isolate left renal artery and clamped for 30 min. Subsequently the wound in the

abdomen was sutured, povidone iodine ointment was applied and animals were housed individually in cages. The rats were then allowed to recover and dosing was continued as mention above till day 30th day after surgery. Blood and urine samples were collected on 0, 10, 20 and 30th day after surgery for biochemical and renal function estimations.

Measurement of serum biochemical and renal function parameters (Sharma *et al.*, 2006):

On selected days, rats were placed in metabolic cages (Techniplast, Milan, Italy) and 24 h urine was collected. On each of these days, rats were anaesthetized by anaesthetic ether and blood was withdrawn by retro-orbital puncture using micro capillary tubes. Serum was obtained by centrifuging the blood at 7000 rpm. at 4°C. The creatinine levels and Blood Urea Nitrogen (BUN) were measured in serum and 24 h urine samples, respectively. Creatinine clearance (CCr) was calculated by reported method (Cockcroft and Gault, 1976):

$$\text{Creatineclearance (CCr)} = \frac{\text{Urinecreatinine (mg dL}^{-1}\text{)} \times 24(\text{h}) \times \text{urine volume (mL)}}{\text{Serum creatinine (mg dL}^{-1}\text{)} \times 1440}$$

Histopathology of kidneys in diabetic rats (Melin *et al.*, 1997; Melin *et al.*, 2002):

On 30th day, after collection of urine and serum, the animals were sacrificed. Their kidneys were removed, stored in 10% formalin until dissected. At the time of dissection, kidneys were divided into two halves (parallel to major axis) and washed. These tissues were processed for 12 h using isopropyl alcohol and xylene and embedded in paraffin. Paraffin embedded tissues were sectioned (5 µm thickness) and stained using haematoxylin and eosin (H and E). Histological changes in the glomeruli, tubules, interstitium and blood-vessels were recorded for each specimen by photomicrographic examination with light microscope (Olympus, USA) fitted with camera (Nikon E200). Renal changes were scored using a scale of none (-), mild (+), moderate (++) and severe (+++) damage.

Statistical analysis: Data was expressed as Mean ± SEM for each biochemical parameter. Data for CCr and BUN was separately analyzed by two way ANOVA followed by Bonferroni Posttest for each model. p<0.05 was considered statistically significant.

RESULTS

Effect of IND01 on creatinine clearance (CCr) in alloxan-induced DN in rats without I/R: Alloxan (160 mg kg⁻¹,

Table 1: Effect of IND01 on creatinine clearance (CCr) and blood urea nitrogen (BUN) in alloxan-induced diabetic rats without ischemia reperfusion (I/R)

CCr (mL min ⁻¹)	Day 0	Day 15	Day 30
Non-diabetic	1.053±0.032	1.057±0.024	1.000±0.06
Diabetes control	0.508±0.009####	0.402±0.09####	0.387±0.009####
Diabetes+Pioglitazone (10)	0.509±0.0125	0.559±0.019**	0.733±0.021***
Diabetes+IND01 (50)	0.529±0.032	0.499±0.023	0.548±0.02**
Diabetes+IND01 (100)	0.535±0.016	0.509±0.012	0.586±0.017***
Diabetes+IND01 (200)	0.526±0.0176	0.573±0.024***	0.635±0.014***
BUN (mg dL ⁻¹)	Day 0	Day 15	Day 30
Non-diabetic	20.190±0.68	18.080±0.92	15.001±0.91
Diabetes control	42.140±2.76####	47.970±2.39####	46.060±0.36####
Diabetes+Pioglitazone (10)	41.590±0.81	35.770±1.24***	30.510±0.515***
Diabetes+IND01 (50)	43.620±2.25	52.630±1.84	36.630±2.19***
Diabetes+IND01 (100)	42.460±2.08	51.460±1.25	34.410±1.98***
Diabetes+IND01 (200)	42.350±1.58	48.900±1.48	31.200±1.77***

*Values in the bracket are dose in mg kg⁻¹, per oral, All rats from all groups except normal group were made diabetic with alloxan (160 mg kg⁻¹, i.p.) treatment, Values are expressed as mean±SEM. (n = 6), Data was analyzed by two-Way ANOVA followed by Bonferroni Posttest for each parameter separately. # p<0.5, ## p<0.01 and #### p<0.001 as compared with non-diabetic rats; *p<0.05, **p<0.01, ***p<0.001 as compared to diabetic control rats on respective days

Table 2: Effect of IND01 on creatinine clearance (CCr) in alloxan-induced diabetic rats with ischemia reperfusion (I/R)

CCr (mL min ⁻¹)	Day 0	Day 10	Day 20	Day 30
Non-diabetic	1.03±0.08	1.05±0.19	1.03±0.19	1.06±0.01
Diabetes Sham	1.38±0.10	1.28±0.05	1.23±0.06	1.15±0.06
Diabetes+I/R	0.35±0.04####, \$\$\$	0.28±0.07####, \$\$\$	0.26±0.06####, \$\$\$	0.18±0.01####, \$\$\$
Diabetes+I/R+Insulin (8 IU)	1.28±0.08***	1.10±0.08***	0.69±0.05***	0.40±0.01
Diabetes+I/R+IND01 (50)	0.42±0.05	0.34±0.06	0.32±0.08	0.21±0.02
Diabetes+I/R+IND01 (100)	0.83±0.05***	0.54±0.09	0.53±0.09	0.29±0.02
Diabetes+I/R+IND01 (200)	1.07±0.11***	0.69±0.08**	0.59±0.05*	0.33±0.01

All rats from all groups except normal group were made diabetic with alloxan (70 mg kg⁻¹, i.v.) treatment, Values are expressed as mean±SEM. (n = 6), Data was analyzed by two-Way ANOVA followed by Bonferroni Posttest for each parameter separately. # p<0.5, ## p<0.01 and #### p<0.001 as compared with non-diabetic rats; \$ p<0.05, \$\$ p<0.001, \$\$\$ p<0.001 as compared with diabetic sham rats; *p<0.05, **p<0.01, ***p<0.001 as compared to diabetes with I/R rats on respective days

I.p.) injection significantly reduced CCr as compared with non-diabetic (normal) rats within 48 h. This fall suggests induction of kidney dysfunction by alloxan. The fall in CCr was sustained during the study period of 30 days (Table 1). Daily once treatment of pioglitazone (10 mg kg⁻¹, p.o.) and IND01 (200 mg kg⁻¹, p.o.) in alloxan-induced rats (group 2) improved CCr from 15th days of treatment. Lower dose of IND01 (50 and 100 mg kg⁻¹, p.o.) treatment improved CCr on day-30.

Effect of IND01 on Blood Urea Nitrogen (BUN) in alloxan-induced DN in rats without I/R: Sustained increase (p<0.001) in BUN levels were observed in alloxan-treated (diabetic) rats as compared normal (non-diabetic rats). Moreover, BUN levels were significantly higher on day 30 from baseline (day 0) (Table 1). Pioglitazone treatment significantly (p<0.001) lowered BUN levels from day-15 onwards. IND01 treatment showed significant decrease in BUN levels only after 30 days of treatment (Table 1).

Effect of IND01 on creatinine clearance (CCR) in alloxan-induced DN in rats with I/R: Alloxan (160 mg kg⁻¹, i.p.) pretreatment brought significantly sharp fall in CCr compared with non-diabetic (normal) as well as diabetic sham control rats within 48 h. This fall suggests induction of reduced kidney function in diabetic

rats. The fall in CCr was sustained in the study period of 30 days. Daily treatment of Insulin (8 IU, s.c.) and IND01 (100 and 200 mg kg⁻¹, p.o.) to renal I/R in diabetic rats showed improved CCr form at baseline (48 h. after diabetes induction). Insulin produced significant CCr improvement compared with diabetes with I/R rats till day 20 of study. Improvement in IND01 treated rats did not sustain beyond 10 days (dose 200 mg kg⁻¹). On day-30, neither Insulin nor IND01 showed significant improvement in CCr compared with diabetes rats with I/R (Table 2).

Effect of IND01 on Blood Urea Nitrogen (BUN) in alloxan-induced DN in rats with I/R: Sustained increase (p<0.001) in BUN levels in alloxan treated rats were found as compared normal (non-diabetic rats) and BUN levels were significantly higher form baseline (day 0) to 30-days (Table 3). Pioglitazone treatment significantly (p<0.001) lowered BUN levels from day-15 onwards. IND01 treatment showed significant decrease in BUN levels only after 30 days of treatment (Table 3). At baseline (48 h. after diabetes induction), BUN levels in ischemic-injured diabetic rats were found as significantly higher (p<0.001) compared to non-diabetic and diabetic sham control rats. Significantly (p<0.001) higher BUN levels were observed in ischemic-injured diabetic rats during the

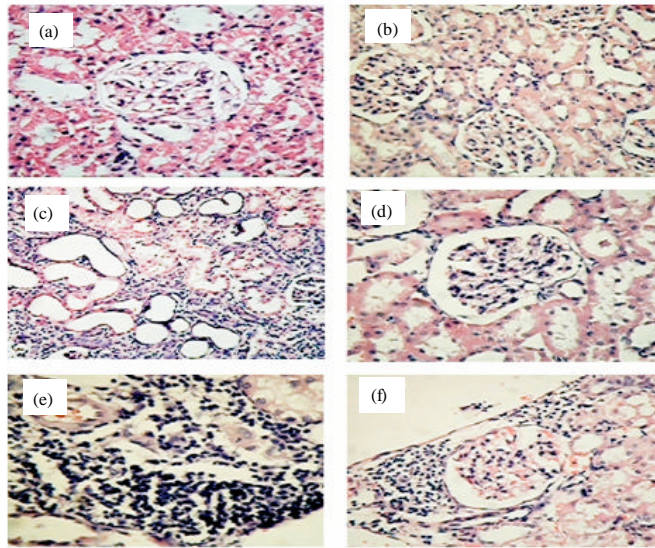


Fig. 1(a-f): Photomicrograph of section of Kidneys showing glomerulus of alloxan-induced diabetic rats after 30 days of treatment with (a) Normal (b) Alloxan (160 mg kg⁻¹, i.p.) (c) Pioglitazone (10 mg kg⁻¹, p.o.) (d) IND01 (50 mg kg⁻¹, p.o.) (e) IND01 (100 mg kg⁻¹, p.o.) and (f) IND01 (200 mg kg⁻¹, p.o.), Sections were stained with hematoxylin and eosin (HE) at magnification of 100 X

Table 3: Effect of IND01 on blood urea nitrogen (BUN) in alloxan-induced diabetic rats with ischemia reperfusion (I/R)

BUN (mg dL ⁻¹)	Day 0	Day 10	Day 20	Day 30
Non-diabetic	25.10±0.42	28.28±0.48	31.60±0.38	32.00±0.39
Diabetes Sham	26.10±0.48	29.28±0.48	32.60±0.38	32.32±0.39
Diabetes+I/R	31.32±0.63	45.44±7.23 ^{####, \$\$\$}	45.12±3.99 ^{###, \$}	46.81±1.91 ^{###, \$\$}
Diabetes+I/R+Insulin (8 IU)	25.01±0.51	29.30±0.42 ^{***}	33.70±0.33 [*]	33.00±0.35 ^{**}
Diabetes+I/R+IND01 (50)	31.00±1.71	40.20±7.00	43.00±3.83	45.00±2.70
Diabetes+I/R+IND01 (100)	30.89±1.42	40.00±6.38	39.00±3.00	38.00±1.09
Diabetes+I/R+IND01 (200)	31.21±1.22	39.21±6.46	34.97±0.66	34.63±1.48 [*]

All rats from all groups except normal group were made diabetic with alloxan (70 mg kg⁻¹, i.v.) treatment. Values are expressed as mean±SEM. (n = 6), Data was analyzed by two-Way ANOVA followed by Bonferroni Posttest for each parameter separately, # p<0.05, ## p<0.01 and ### p<0.001 as compared with non-diabetic rats, \$ p<0.05, \$\$ p<0.001, \$\$\$ p<0.001 as compared with diabetic sham rats, *p<0.05, **p<0.01, ***p<0.001 as compared to diabetes with I/R rats on respective days

study period of 30 days. Insulin (8 IU, s.c., once a day) treatment did not show significant rise in BUN as shown by ischemic-injured diabetic rats (Table 2). On 30-days of pre-treatment, IND01 (50 or 100 mg kg⁻¹, p.o., once daily), did not show any reduction in BUN in Ischemic-injured rats. However, at dose of 200 mg kg⁻¹, p.o., IND01 showed mild (p<0.05) reduction from raised BUN levels as compared with ischemic-injured diabetic rats. Effect of IND01 on histology of kidneys in alloxan-induced DN in rats without I/R: The histological observations of sections of kidneys of alloxan treated rats showed hyperplasia of mesangium, glomerular basement membrane thickening, tubular atrophy and interstitial inflammation of mild to moderate intensity confirming induction of nephropathy (Table 4 and Fig. 1). These changes were absent in rats treated with pioglitazone and IND01 (200 mg kg⁻¹) which

confirms renoprotective action as well as antihyperglycemic effects. However, lower doses of IND01 (50 and 100 mg kg⁻¹), caused certain degree (although mild in intensity) of hyperplasia of mesangium and glomerular basement membrane enlargement. Except normal (non-diabetic) rats, moderate tubular atrophy, tubular dilation and interstitial inflammation was noted in all the kidney samples (including kidneys of IND01 and pioglitazone treated diabetic rats), which suggest irreversible nature of tubular atrophy in diabetic nephropathy.

Effect of IND01 on kidney histopathology in alloxan-induced DN in rats with I/R: The histological observations of sections of kidneys of ischemic-injured diabetic rats showed moderate to severe (++ to+++)

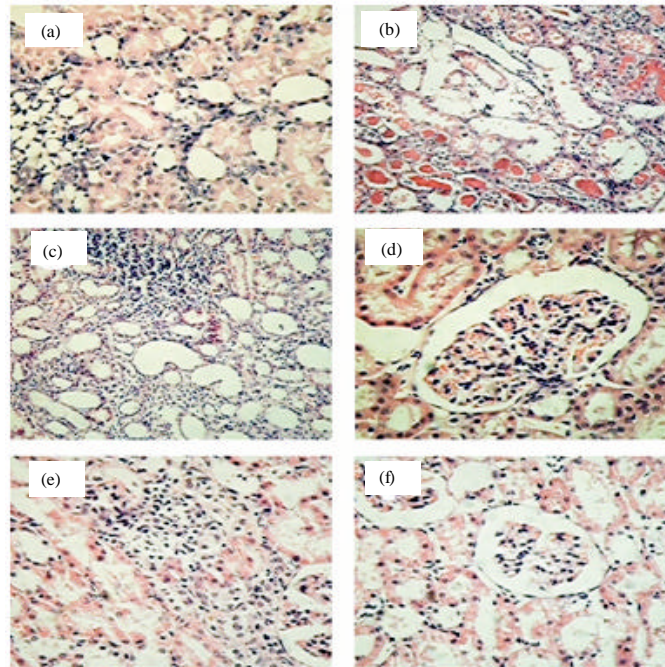


Fig. 2(a-f): Photomicrograph of section of Kidneys showing glomerulus on day-30 in rats alloxan-induced diabetic rats without renal I/R in diabetes sham (a) and with I/R (b), respectively are shown. The kidneys of rats with 7-day pre - and 30 day post I/R treatment with (c) Insulin (8 IU, s.c.), (d) IND01 (50 mg kg⁻¹, p.o.), (e) IND01 (100 mg kg⁻¹, p.o.), (f) IND01 (200 mg kg⁻¹, p.o.) in diabetes with renal I/R induction are also shown. Sections were stained with hematoxylin and eosin (HE) at magnification of 100 X

Table 4: Effect of IND01 on histopathology of sections of kidney in early DN in alloxan-induced diabetic rats

Treatment	Hyperplasia of mesangium	Glomerular basement membrane enlargement	Tubular atrophy	Interstitial inflammation
Non-diabetic	--	--	--	--
Diabetes control	++	+	++	++
Diabetes + Pioglitazone (10)	--	--	++	++
Diabetes + IND01 (50)	++	+	++	++
Diabetes + IND01 (100)	+	+	++	++
Diabetes + IND01 (200)	+	--	++	++

All rats from all groups except normal group were made diabetic with alloxan (160 mg kg⁻¹, i.p.) treatment, +:- Mild ++:- Moderate, +++:- Severe -- :- Absent

Table 5: Effect of IND01 on histopathology of sections of kidney in I/R induced in alloxan-induced diabetic rats

Treatment	Glomerular cellularity	Glomerular matrix	Tubular necrosis with dilatation	Interstitial inflammation	Interstitial fibrosis
Non-diabetic	++	+	++	+	+
Diabetic sham	++	+	+++	+	+
Diabetes+I/R	--	-	+	+	+
Diabetes+I/R+Insulin (8 IU)	--	-	+	+	+
Diabetes+I/R+IND01 (50)	+	-	+	+	+
Diabetes+I/R+IND01 (100)	+	-	+	+	+

All rats from all groups except normal group were made diabetic with alloxan (70 mg kg⁻¹, i.v.) treatment, +: Mild ++: Moderate +++: Severe, -- : Absent figure legends

glomerular cellularity, glomerular matrix formation, tubular necrosis with dilatation, interstitial inflammation and fibrosis. Diabetic sham control rats also showed mild changes in kidney morphology (Table 5 and Fig. 2). In our

study, insulin treatment caused complete prevention in kidney sections with respect to glomerular cellularity and matrix formation during 30 days of period whereas IND01 treatment prevented these changes partially. Mild to

moderate tubular necrosis with dilatation, interstitial inflammation and fibrosis were observed in all the kidney sections, which suggest irreversible nature of tubular atrophy. These changes were severe in Ischemic-injured diabetic rats. The rats treated with Insulin or IND01 for 30 days showed only mild changes compared to rats having diabetes and I/R (Table 5).

DISCUSSION

One of the specific chronic complications is represented by the finding of diabetic nephropathy that is characterized by proteinuria, frequent hypertension and slow gradual alteration of renal functions. In a narrow sense of the word, diabetic nephropathy is referred to as microangiopathic impairment of kidneys.

Fenugreek as a diet supplement causes a marked decrease in symptoms of diabetes in terms of polydipsia, polyuria, urine sugar, renal hypertrophy and glomerular filtration rate (Shetty and Salimath, 2009). In the present study, we investigated effects of reconstructed composition of *Trigonella foenum-graecum* L. seeds. Each ingredient in fenugreek extract has different chemical constituents with complex molecular formulae, which must be beneficial for different aspect of diabetes management (Hassanzadeh *et al.*, 2011). Another challenge is consistency in quality and efficacy of composition (Ansari and Inamdar, 2010; Sarwar *et al.*, 2011). The present study was an attempt to study optimized composition for the therapeutic effects in the animal model of renal complication of DM. IND01 is reconstructed composition which contains active ingredients of fenugreek seeds in same proportion as they found in nature.

In the present study, alloxan with or without renal ischemic-injury produced severe biochemical or histological in rats kidneys. CCr levels were decreased and BUN levels were increased in diabetic as well as renal I/R with diabetic rats. These results are consistent with earlier reports on alloxan model (Spadella *et al.*, 1998) and effect of pioglitazone (Stendig-Lindberg, 1992).

In the present study, consistent decrease in CCr in the diabetic rats with and without renal I/R was observed. IND01 showed dose-dependent improvement in CCr in alloxan induced diabetes rats after 30 days of treatment. In the diabetic rats with renal I/R, pretreatment of IND01 for 7 days could offer only moderate reversal of CCr. The protective effects of IND01 on CCr were found to decline over the period of 30 days (Table 3). On the other hand, administration of insulin (8 IU, s.c.) prior to I/R in diabetic rats offered consistent improvement in CCr during the study period in diabetic rat with I/R (Table 3).

CCr is an effective means of assessing renal function and useful indicator of glomerular filtration rate (GFR). CCr is the removal of creatinine from the body and defined as the volume of blood plasma that is cleared of creatinine per unit time. The pathophysiology of DN involves glucose that binds irreversibly to proteins of kidneys and AGEs, that can form complex cross-links over years of hyperglycemia. DN can cause renal damage by stimulation of growth and fibrotic factors via receptors for AGEs. Increased glomerular capillary pressure occurs early in diabetes and is associated with hyperfiltration at the glomerulus. The glomerular mesangium expands, initially by cell proliferation and then by cell hypertrophy. Increased mesangial stretch and pressure, as well as high glucose levels, can stimulate this expansion. Besides, the role of glycosylation of proteins is involved in alloxan induced DN model in animals (El-Mekawi *et al.*, 1993). Higher level of glycosylation of urinary proteins after alloxan induced diabetes rats after 7 days of treatment had been reported earlier (El-Mekawi *et al.*, 1993). Therefore, ability of any compound in reversing renal dysfunction point to good control of glycosylation of protein. In present study, the protection from renal dysfunction by IND01 in early-stage diabetic nephropathy can be attributed to probable fall in AGEs and improved glycemic control. Fenugreek has been reported to improve glycemic control and glycated haemoglobin (HbA1c) status in diabetes patients (Gupta *et al.*, 2001; Kassaian *et al.*, 2009) and animals (Devi *et al.*, 2003; Xue *et al.*, 2007). Galactomannan from fenugreek is especially reported for this purpose (Srichamroen *et al.*, 2008).

Similar effects were noted in terms of BUN measurements in the present study. IND01, pioglitazone and insulin showed significant improvement of BUN levels in the doses tested against diabetes with or without renal I/R. However, onset of action to achieve the protection was quickest for Insulin (48 h after diabetes with renal I/R) followed by pioglitazone (15 days in diabetes without I/R) and IND01 (30 days in both models). IND01 at a dose as low as 50 mg kg⁻¹, p.o., showed potent protective effects in diabetic rats (without I/R). In presence of renal I/R in diabetic rats, IND01 could only produce mild effects at highest dose (200 mg kg⁻¹) after 30 days of treatment. This observation is consistent with earlier report that insulin offers renal protection from I/R when improved metabolic control is achieved before renal I/R (Melin *et al.*, 2002). The control of hyperglycemia before I/R is important for renoprotective action of insulin and IND01 in diabetes in presence of renal I/R.

BUN measurement is another kidney function assessment test. Urea is a by-product of protein metabolism and is formed in the liver. Urea is excreted in

urine and undergoes tubular reabsorption. Because of tubular atrophy in DM, tubular reabsorption of BUN is affected. Therefore, high amount of BUN in the diabetic rats with or without renal I/R indicate impairment of tubular reabsorption which was also observed in our study.

The changes in kidneys that can be evaluated histopathologically depend on the duration of diabetes and how diabetes was treated. In short-lasting untreated diabetes, character of diabetic nephrosis is found to be manifested microscopically in diabetic patients (Farquhar *et al.*, 1959; Meoro *et al.*, 1999; Rosai and Ackerman, 2004) as well as alloxan-induced rats (Bartosikova *et al.*, 2003). In the present study, sections of kidneys of diabetic rats with and without I/R showed cellularity, glomerular matrix formation, tubular necrosis with dilatation, interstitial inflammation and fibrosis. The intensity of kidney damage was severe (+++) with I/R whereas moderate damage (++) was observed without I/R. Daily oral administration of IND01 (50-200 mg kg⁻¹) for 30 days seems to provide moderate (++) protection from histological abnormalities in kidneys of early experimental DM (without I/R). However, such protection was not offered by IND01 in diabetes with I/R model.

Alloxan is known to produce tubular atrophy, interstitial inflammation, hyperplasia of mesangium and glomerular basement membrane enlargement. These renal changes are because of high glucose levels present for longer period in the diabetic rats. There is growing evidence that hyperglycemia results in altered renal oxygen metabolism and decreased renal oxygen tension and that these changes are linked to altered kidney function (Shafir and Gutman, 1993). Current evidence suggests the selective cytotoxicity of alloxan is because of efficient uptake, oxidant production by redox coupling of alloxan with intracellular reductant (ascorbate and thiols) coupled with low levels of glutathione peroxidase in the islets (Malaisse, 1982).

Much of the evidence concerning the role of oxidative stress in the induction of DM comes from the studies of alloxan which produces diabetes in experimental animals (Wolff, 1993; Balkis *et al.*, 2008). Alloxan selectively destroys the islets of Langerhans by oxidant production, enhances lipid peroxidation and susceptibility to oxidative stress associated with depletion of antioxidants in liver, kidney and pancreas (Anuradha and Ravikumar, 2001). Alloxan toxicity *in vitro* and *in vivo* can be inhibited by many free radical scavengers and lipid-soluble antioxidants (Malaisse, 1982; Wolff, 1993). Therefore, alloxan-induced DM served as pathological bio-model for testing a substance with supposed antioxidative activity *in vivo*.

Fenugreek seeds have been shown to have potent antioxidant effects and protect many vital organs in the body against oxidative stress induced by alloxan (Ravikumar and Anuradha, 1999; Anuradha and Ravikumar, 2001; Al-Wabel *et al.*, 2008; Al-Matubsi *et al.*, 2011; Premanath *et al.*, 2011). Therefore, antioxidant potential can also envisaged as possible mechanism for renal protection shown by IND01 in the present study. However, antioxidant activity in fenugreek seeds was primarily attributed to the presence of flavonoids and polyphenols (Dixit *et al.*, 2005). The absence of flavonoids and/or polyphenols in test composition (IND01) in present study pointed towards mechanisms other than anti-oxidant potential.

On the other hand, sustained hyperglycemia caused by alloxan administration results into many biochemical and histological abnormalities and lead to nephropathy in rabbits (Winiarska *et al.*, 2008), mice (Nordquist *et al.*, 2008) and rats (Macedo *et al.*, 2007). Further, chronically hyperglycemic alloxan diabetic rats showed reduced glomerular filtration rates renal plasma flow (p-aminohippurate clearance) and extracellular fluid volume associated with urinary Na⁺ losses which eventually reduces GFR (Di Loreto *et al.*, 2004). It has been postulated that glomerular hyperfiltration or elevated GFR in early diabetes may eventually cause glomerular damage, leading to a progressive loss of renal function (diabetic nephropathy). Diabetic rats with long-term moderate hyperglycemia, however, do not develop characteristic glomerular lesions of human diabetic nephropathy and, in fact, develop only minimal glomerular injury even after 1-year of diabetes (O'Donnell *et al.*, 1988). Long-term diabetes in animals could accelerate the formation of the intercellular matrix of glomerular loops in proliferative glomerulitis in rabbits, resulting in accelerated glomerulosclerosis (Wanibuchi *et al.*, 1991) and further worsens the nephropathy. Thus, although the diabetic rat with moderate hyperglycemia (induced by alloxan or streptozotocin) may be useful to study the mechanisms in early diabetes, it may not be an appropriate model of renal failure in IDDM (O'Donnell *et al.*, 1988).

On the other hand, I/R combined with hyperglycaemia can mimic important mechanisms to develop ESRD and/or diabetic nephropathy in diabetic animals (Melin *et al.*, 2003). Hyperglycemia is known to aggravate the renal injury and causes acute renal ischemia in the rat (Podrazik *et al.*, 1989). An exceptional susceptibility to unilateral renal I/R injury resulting in inflammation, fibrosis, atrophy of the kidney and ESRD had been demonstrated in the diabetic rat (Melin *et al.*, 2002). Studies also showed that a brief I/R results in a progressive injury leading to end-stage renal failure (similar to ESRD) in diabetic animals

(Melin *et al.*, 1997; Melin *et al.*, 2002). In DM animals renal I/R caused massive induction of apoptosis in the renal medulla after six hours as well as inflammation, fibrosis, renal atrophy and anuria within four weeks (Melin *et al.*, 2002). Several mechanisms have been suggested to explain why hyperglycaemia increases I/R injury. Intracellular oxidative has been proposed as a unifying explanation for most metabolic alterations in diabetes (Brownlee, 2001) I/R is also a state where oxidative stress has been implied in case of renal ischemic injury (Chien *et al.*, 2001). It could be speculated that the combined oxidative stress from these two sources (diabetes and I/R) could be especially harmful. The increased inflammatory response after I/R in diabetes may play a role in increased sensitivity to I/R in diabetes (Panis *et al.*, 1996). The beneficial effects of IND01 on CCr in both the models and mild reduction shown in basement membrane enlargement (in diabetic I/R model) were observed in present study. The results support hypothesis for antiinflammatory and/or antioxidant activity as potential mechanism of renoprotective action of IND01 Besides the glomerular changes, interstitial fibrosis and infiltration of inflammatory cells are important features of diabetic nephropathy (Bohle *et al.*, 1991). In fact, the degree of tubulointerstitial injury is as closely related to the decline in function as are the glomerular alterations (Bader *et al.*, 1980). The tubulointerstitial fibrosis and infiltration of inflammatory cells observed in diabetic rats after renal I/R share similarities with diabetic nephropathy (Melin *et al.*, 1997). In the past, aqueous extract of fenugreek has been reported to have antiinflammatory activity (Vyas *et al.*, 2008; Subhashini *et al.*, 2011). Fenugreek leaves extract has been shown potent anti-inflammatory effects in animals of inflammation (Ahmadiani *et al.*, 2001). Because of presence of alkaloids and absence flavonoids, saponins, or steroids in fenugreek leaves extract, authors suggested alkaloid as a responsible constituent for antiinflammatory potential of fenugreek. Therefore, the protective effect shown by IND01 can be partly attributed by presence of an alkaloid constituent, trigonelline. In the past, many natural and synthetic analogs of leucine (an amino acid) also showed antiinflammatory potential (Somasundaram *et al.*, 1983; Turek *et al.*, 1991; Bator *et al.*, 1992; Serebruany *et al.*, 1995). Therefore, possible contribution of 4-hydroxyisoleucine in the renoprotective action (through antiinflammatory mechanism) against diabetes cannot be ruled out.

CONCLUSION

In conclusion, IND01 showed potential for renal protection against diabetic rats with or without renal I/R. The effects seem to be mediated through effective glycemic control.

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