Protective effect of *Monascus* fermented rice against STZ-induced diabetic oxidative stress in kidney of rats

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Revised: 21 September 2013 / Accepted: 30 September 2013 / Published online: 11 October 2013
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Abstract In the present study, anti-diabetic activity and nephroprotective effect of MMFR was evaluated by using STZ-induced diabetic rats. Administration of MMFR at 100 and 200 mg/kg bw showed significant ($P < 0.01$) anti-hyperglycemic activity by lowering blood glucose level, HbA_{1C} and increasing body weight. Altered lipid profiles in diabetic rats were restored to normal level on treatment with MMFR and showed significant ($P < 0.01$) decrease in the elevated levels of biochemical parameters. MMFR produced significant ($P < 0.01$) improvement in antioxidant levels in kidney. Food safety of MFR produced by using mutant *Monascus purpureus* 254 (MMFR) was evaluated for genotoxicity and oral acute toxicity. In Ames mutagenicity assay MMFR doesn't showed any toxicity to the test strain *S. typhimurium* till 5 mg/plate. Acute toxicity study also recorded no toxicity till the dose of 5,000 mg/kg bw. The study concluded that MMFR, not only possess anti-diabetic activity but also prevents nephropathy and hypercholesterolemia due to diabetes.

Keywords *Monascus* fermented rice (MFR) · Diabetic nephropathy · Citrinin · STZ · Diabetes mellitus

Introduction

Diabetes mellitus is a metabolic disorder due to insufficient secretion of insulin, its action, or both (Singh et al. 2008). Diabetes mellitus is characterized by hyperglycemia resulting in various complications due to oxidative stress, induced by free radical generation. Chronic hyperglycemia results in glucose oxidation and lipid peroxidation, a main source of free radicals (Ceriello and Testa 2009). In Diabetes mellitus reduction in the levels of antioxidant enzymes were also reported. Oxidative stress due to prolonged hyperglycemia leads to various relative disorders, among which diabetic nephropathy is one of the main causes for increase in mortality in diabetic patients (Baynes 1991).

Filamentous fungi play an important role in production of food and health care products. The fungi belong to genera *Monascus* were used in Asian countries particularly in China as a source of coloring agents for food. Red yeast rice produced by fermenting rice with *Monascus* spp. was used as a medicine to strengthen spleen, promote digestion and blood circulation in China (Heber et al. 1999). Various studies also reported the therapeutic potential of red yeast rice against cholesterol (Rajasekaran and Kalaivani 2011), osteoporosis (Ricky and Bakr 2008) and cancer (Ho et al. 2010). In our earlier study we have reported anti-diabetic activity of *Monascus* fermented rice (MFR), produced by fermenting rice with *Monascus purpureus* MTCC 1090 (Rajasekaran et al. 2009).

The presence of citrinin in MFR is a main cause of its adverse effect. Citrinin, a mycotoxin was proved to have nephrotoxic and neurotoxic effect on consumption (Flajs and Peraica 2009; Gupta et al. 1984). In this study, we used MFR produced by using citrinin free mutant *Monascus purpureus*. Though the studies indicated anti-diabetic activity of MFR in STZ-induced animal models, its nephroprotective effect under diabetic conditions has not yet been evaluated. The present

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study was thus initiated with the aim of evaluating systematically the various effects of MFR in STZ-induced diabetes and its related complications in rat models.

Materials and methods

Materials

The MFR used for the study was produced by fermenting rice IR-532-E-576 with citrinin free mutant *Monascus purpureus* 254 (unpublished data), developed in our laboratory and designated as MMFR. The fermented rice was sterilized by autoclaving at 121 °C for 20 min before its administration in animals.

S. typhimurium TA98, TA100 and TA102 were obtained from Microbial type culture collection (MTCC), Chandigarh, India.

Chemicals used for the study were purchased from Sigma Chemicals, India. Equipments such as Autoclave, BOD incubator, Hot air oven, Laminar air flow and Tissue homogenizer were from Remi Laboratory Instruments, India, Semi-auto analyzer, Photometer 5010_{v51}, Germany and Colony counter, Deep vision instruments pvt ltd, India were used.

Experimental animals

Wistar rats of either sex, weighing 200–250 g were used in this study. The animals were procured from the animal house, Kovai Medical Centre Research and Educational Trust (KMCRET), Coimbatore, Tamil Nadu after getting the approval from Institutional Animal Ethical Committee (KMCRET/PhD/1/09 valid till October 2010). The animals were treated in accordance with the Institutional guidelines and recommendations for the proper care and use of laboratory animals.

Determination of acute oral toxicity

Acute oral toxicity study was performed as per Organization for Economic Cooperation and Development (OECD) guideline 423 method (OECD 2001). For oral acute toxicity study MMFR was powdered and prepared with distilled water. Six animals were divided into two groups ($n=3$). The overnight fasted animals were administered with MMFR in a single dose (5, 50, 300, 2,000 and 5,000 mg/kg bw) in 0.5 mL of distilled water. After administration of MMFR, food was withheld for 2 h. Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 23 h, with special attention given during the first 4 h, and daily thereafter, for a total period of 14 days to assess the appearance of delayed toxicity.

Ames bacterial mutagenicity assay

The non-mutagenicity of MMFR was evaluated by using Ames assay as per Maron and Ames (1983) method and OECD (471) guideline (OECD 1997) by plate incorporation method in the presence and absence of metabolic activation system (S9 liver fraction). To carry out the assay the histidine-dependent strains of *S. typhimurium* TA98, TA100 and TA102 were used. The positive controls were maintained using standard mutagens such as daunomycin (6 µg/plate) for TA98, sodium azide (1 µg/plate) for TA100 and phenyl hydrazine (20 µg/plate) for TA102.

MMFR was tested for its mutagenic property at six different concentrations of 0.5, 1, 2, 3, 4 and 5 mg of MMFR/plate. The various concentrations of powdered MMFR, 500 µL of histidine-biotin potassium chloride (KCl) were added to 2 mL molton top agar and then mixed with 100 µL of bacterial culture (1×10^9 cells/mL). The contents were mixed gently and thoroughly, finally poured on to a plate containing minimal glucose agar and spreaded evenly. After top agar solidified, the plates were incubated at 37 °C for 48 h. Negative controls were also maintained by adding water instead of MMFR or mutagen.

The influences of metabolic activation were tested by adding 500 µL of liver microsomal fraction (S9) mixture. S9 was prepared from Sprague Dawley rat (200 g). The rat was treated with 0.1 % phenobarbital in water for 4 days. After overnight fasting, the animals were killed by decapitation, the liver was removed and homogenized, aseptically. The activation mixture was prepared by mixing 50 µL of the S9 fraction, containing 0.25 mL phosphate buffer (0.2 M, pH 7.4), 20 µL nicotinamide adenine dinucleotide phosphate (sodium salt) (0.1 M), 2.5 µL glucose-6-phosphate (1 M) and 10 µL of 1.65 M magnesium chloride and 0.4 M potassium chloride. Metabolic activation experiments were conducted by adding 0.5 mL of rat liver S9 fraction to top agar (Ames et al. 1973). After incubation, histidine revertants colonies were counted using colony counter.

In vivo evaluation of anti-diabetic activity of MMFR

Induction of experimental diabetes

Diabetes was induced in overnight fasted rats by intraperitoneal administration of streptozotocin (STZ) at the dose of 60 mg/kg bw after dissolving in freshly prepared cold citrate buffer (0.1 M, pH 4.5) (Singab et al. 2005). The rats were provided with 5 % dextrose solution after 6 h of STZ administration for next 24 h to prevent STZ induced fatal hypoglycemia (Tanko et al. 2008). After 72 h of induction diabetes was confirmed by measuring tail vein blood glucose levels using glucose meter (Glucocard™ 01-mini, Arkray Factory, Inc., Japan) by glucose oxidase-peroxidase method. Diabetic

rats were kept for 14 days under standard laboratory condition for the stabilization of blood glucose level (Vogel and Vogel 2002). After 14 days, blood glucose was determined and only animals with a blood glucose level greater than 300 mg/dL were selected for the study (Abeeleh et al. 2009).

Experimental design for anti-diabetic activity

The rats were divided into five groups ($n=6$). The group 1: control rats received propylene glycol (5 mL/kg bw); group 2: STZ-induced diabetic rats received propylene glycol (5 mL/kg bw); group 3: diabetic rats received MMFR 100 mg/kg bw; group 4: diabetic rats received MMFR 200 mg/kg bw and group 5: diabetic rats received glibenclamide 5 mg/kg bw (Safiyeh et al. 2007). The vehicle, MMFR and glibenclamide were administered to the respective group animals for 21 days. The fasting body weight, blood glucose level were estimated on 0, 7, 14 and 21 day periodically. On 21st day, overnight fasted animals received respective treatment and after 1 h of treatments all animals were anaesthetized with ketamine (100 mg/kg, i.p.); blood sample was collected through retro-orbital plexus puncture and stored in with or without disodium ethylene diamine tetra acetate for the biochemical parameters estimation.

Measurement of biochemical parameters

Blood glucose, hemoglobin (Hb) and glycosylated hemoglobin (HbA_{1C}) were estimated using whole blood. The high density lipoprotein (HDL), total cholesterol, triglycerides, total protein, creatinine, uric acid, blood urea nitrogen (BUN), urea, serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) were estimated in serum by kits specific for the test using semi-autoanalyzer. The serum insulin was determined by radioimmunoassay method (Flier et al. 1979). The low density lipoprotein (LDL) and very low density lipoprotein (VLDL) levels were calculated by the following equation (Friedewald et al. 1972).

$$VLDL = \text{Triglycerides}/5$$

$$LDL = \text{Total cholesterol} - (\text{HDL} + \text{VLDL})$$

BUN was calculated by using following equation (Nanda Maheswari 2008)

$$BUN = \text{Urea} \times 0.467$$

Determination of in vivo antioxidant levels

Kidney samples were dissected out and washed immediately with ice cold saline to remove blood. The antioxidants parameters such as lipid peroxidation (Fraga et al. 1988) by

measuring thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) (Kakkar et al. 1984), catalase (Sinha 1972), glutathione peroxidase (GPx) (Rotruck et al. 1973) and reduced glutathione (GSH) (Ellman 1959) were estimated.

Statistical Analysis

All the values were expressed as mean $\pm$ SD ($n=6$) and analyzed for analysis of variance (ANOVA) and post hoc Tukey test. Difference between groups was considered significant at $P<0.05$ levels.

Results

Oral acute toxicity studies

The oral administration of MMFR upto the dose 5,000 mg/kg bw was not found to be lethal to animals and no signs of toxicity were observed up to 14 days ($LD_{50}>5,000$ mg/kg). Therefore, biological evaluation was carried out using two dose levels of 100 and 200 mg/kg bw.

Determination of genotoxicity by Ames bacterial mutagenicity assay

To test the mutagenic effect of MMFR, the Ames bacterial mutagenic assay was carried out using three mutant strains of *Salmonella typhimurium* TA98, TA100 and TA102. The results were tabulated in Table 1. The MMFR upto the concentration of 5 mg/plate does not produce any mutagenic effect with or without metabolic activation system (S9) in three test histidine dependent strains. The positive control plates showed three to eight times increase in number of colonies than those of control plates. There is no significant difference between groups of spontaneous revertants and the MMFR treated plates, which indicates that MMFR does not cause any genetic toxicity.

In vivo evaluation of anti-diabetic activity of MMFR

Effect of MMFR on body weight

Characteristic symptom of diabetes such as significant ($P<0.01$) loss of body weight was observed in diabetic control group and all other treated group after the administration of STZ compared to normal rats. Control group animals were found to be stable in their body weight where as diabetic rats showed significant ($P<0.01$) decrease in body weight during 21 days experimental period (Table 2). At the end of experimental period of 21 days diabetic group showed 21.6 % decrease in body weight.

Table 1 Effect of MMFR on *S. typhimurium* spontaneous revertants in the presence (+S9) or absence (–S9) of metabolic activation

Strains	Mixers	Number of revertants (CFU/plate)							
		Negative control	Positive control	MMFR in mg/plate					
				0.5	1	2	3	4	5
TA 98	+S9	52.33±1.5	409.7±1.3 ^a	51.67±1.4 ^b	51.33±1.5 ^b	52.67±3.2 ^b	51±3 ^b	51.67±2.5 ^b	51±1 ^b
	–S9	51.33±1.5	408.7±1.4 ^a	50.67±1.5 ^b	50.33±1.3 ^b	51.67±3.2 ^b	50±3 ^b	50.67±2.5 ^b	50±1 ^b
TA 100	+S9	124.7±1.5	781±1 ^a	124.7±2.5 ^b	127.3±1.1 ^b	125±1 ^b	124.3±2 ^b	125.7±1.5 ^b	124.7±1.1 ^b
	–S9	112.7±1.5	679.3±1.1 ^a	112.7±2.5 ^b	114±2 ^b	113±1 ^b	112.3±2 ^b	113.7±1.5 ^b	112.7±1.1 ^b
TA 102	+S9	280.7±0.5	897.7±6.6 ^a	280.3±1.5 ^b	279.7±2 ^b	280.3±0.5 ^b	279.7±1.5 ^b	279±1 ^b	279.7±2 ^b
	–S9	260.7±0.5	652±2 ^a	260±1 ^b	259.3±2.3 ^b	260.3±0.5 ^b	259±1.7 ^b	259±1 ^b	258.7±2 ^b

value are mean ± SD ($n=3$); ^a $-P<0.01$ positive control compared with negative control and MMFR treated plates, ^b non-significant as MMFR treated plates compared with negative control

Reduction in body weight due to STZ-induced diabetes was reversed by MMFR after 7 days treatment.

Effect of MMFR on blood glucose level

The effect of MMFR on blood glucose level in STZ-induced diabetic rats was shown in Fig. 1. Rats in control group remained euglycemic throughout the experimental period, where as diabetic control rats showed gradual increase in blood glucose level after the administration of STZ from 70.83±0.48 mg/dL to 228.2±0.95 mg/dL. The groups treated with MMFR at the dose 100 and 200 mg/kg bw showed 54.78 % and 29.21 % decrease ($P<0.01$), respectively as compared to diabetic control group. However the effect was lesser than the groups treated with standard drug glibenclamide at the dose of 5 mg/kg bw (58.02 %).

Effect of MMFR on lipid profile

A significant ($P<0.01$) increase in LDL (312 %), VLDL (59.4 %), total cholesterol (17.7 %) and triglycerides (59.4 %) and a significant ($P<0.01$) decrease in HDL (24.2 %) was observed in diabetic control group after administration of STZ when compared to normal control group (Table 2). From these data significant ($P<0.01$) increase in atherogenic index (290 %) was observed, as compared to control group (Table 2). On treatment with MMFR at the dose of 100 mg/kg bw LDL (58.6 %), VLDL (44.8 %), total cholesterol (13.2 %) and triglycerides (44.8 %) showed significant ($P<0.01$) decrease and a significant ($P<0.01$) increase in HDL (34.2 %), which leads to significant ($P<0.01$) decrease in atherogenic index (73.1 %). MMFR at the dose of 200 mg/kg bw showed significant ($P<0.01$) decrease in LDL (65 %), VLDL (15.5 %), total cholesterol (17.8 %) and triglycerides (15.4 %) and significant

Table 2 Effect of MMFR on body weight in STZ-induced diabetic rats

Treatment group	Body weight (g) ± SD				
	Before treatment		After treatment		
	Before STZ diabetic induction	14 day after administration of STZ	7th day	14th day	21st day
Normal control	161.0±1.26	194.0±1.41	195.0±1.41	194.8±1.72	214.3±1.03
Diabetic control	175.7±1.21 ^a	164.3±1.63 ^a	164.0±1.41 ^a	153.8±1.47 ^a	137.7±1.63 ^a
MMFR 100 mg/kg bw	176.0±1.41 ^{ns}	147.2±1.16 ^b	151.2±1.6 ^b	168.5±1.04 ^{b,c}	170.8±1.47 ^{b,c}
MMFR 200 mg/kg bw	183.8±1.47 ^b	142.3±1.03 ^b	150.2±1.94 ^b	160.3±1.03 ^{b, c}	174.2±1.47 ^{b,c}
Glibenclamide 5 mg/kg bw	178±4.38 ^{ns}	135±4.14 ^b	134.7±1.86 ^b	139.0±3.34 ^b	143.8±4.07 ^b

^a $-P<0.01$: Diabetic control compared with normal control group; ^b $-P<0.01$: MMFR 100, 200 mg/kg and Glibenclamide (5 mg/kg) group compared with diabetic control group; ^c $-P<0.01$: MMFR 100, 200 mg/kg compared with Glibenclamide (5 mg/kg) treated group; ^{ns} non significant; All the values were expressed as mean ± SD and analyzed for ANOVA and post hoc Tukey test. Difference between groups was considered significant at $P<0.05$ levels

Fig. 1 Effect of MMFR on blood glucose level in STZ-induced diabetic rats. a- $P<0.01$: Diabetic control compared with normal control group; b- $P<0.01$: MMFR 100, 200 mg/kg and Glibenclamide (5 mg/kg) group compared with diabetic control group; c- $P<0.01$: MMFR 100, 200 mg/kg compared with Glibenclamide (5 mg/kg) treated group; ns-non significant; All the values were expressed as mean $\pm$ SD and analyzed for ANOVA and post hoc Tukey test. Difference between groups was considered significant at $P<0.05$ levels

($P<0.01$) increase in HDL (14.1 %), also resulted in significant ($P<0.01$) decrease of atherogenic index (58 %). whereas standard drug glibenclamide showed significant ($P<0.01$) decrease in LDL (18.6 %) but non-significant decrease in VLDL (3 %), total cholesterol (2.9 %) and triglycerides (3 %) and significant ($P<0.01$) increase in HDL (7.8 %), leads to significant ($P<0.01$) decrease of atherogenic index (20.4 %) as shown in Table 3. The difference between MMFR treated group and Glibenclamide treated group were significant ($P<0.01$), MMFR treated groups showed better management of lipid profile when compared to standard group. MMFR at the dose of 100 mg/kg showed significant ($P<0.01$) decrease in VLDL, total cholesterol and triglycerides and significant ($P<0.01$) increase in HDL when compared to MMFR 200 mg/kg bw.

Effect of MMFR on Hemoglobin, HbA_{1c} and Insulin level

Treatment with MMFR at the dose of 100 and 200 mg/kg bw significantly ($P<0.01$) reduces the HbA_{1c} levels from 12.63 % to 8.6 % and 10.19 % respectively and the HbA_{1c} levels in glibenclamide treated group was 6.68 % (Fig. 2). The

non-significant difference exists in the level of hemoglobin between all the groups, showed that level of hemoglobin is unaffected due to diabetes and its treatment. Insulin level also decreased in diabetic control group. On treatment with MMFR at dose of 100 mg/kg bw the significant ($P<0.01$) increase in the level of Insulin was observed and was comparable to that of standard drug glibenclamide, where as MMFR at the dose of 200 mg/kg bw showed non-significant increase.

Effect of MMFR on biochemical parameters

The level of urea, total protein, creatinine, BUN and uric acid were used to assess the normal function of kidney. The values are shown in Table 4. From the experiments the results showed that there is a significant ($P<0.01$) increase in the level of urea, creatinine and uric acid and significant ($P<0.01$) reduction in total protein in diabetic control group when compared to normal control group. On treatment with MMFR at the dose of 100 mg/kg bw showed significant ($P<0.01$) decrease in the elevated levels of urea, creatinine and uric acid from 224.7 %, 48.6 % and 103.8 %, respectively in diabetic

Table 3 Effect of MMFR on lipid profile in STZ-induced diabetic rats

Lipid Profile	Normal control	Diabetic control	MMFR 100 mg/kg bw	MMFR 200 mg/kg bw	Glibenclamide 5 mg/kg bw
HDL (mg/dL)	52.27 $\pm$ 0.71	39.57 $\pm$ 1.65 ^a	53.12 $\pm$ 1.08 ^{b,c}	45.18 $\pm$ 1.09 ^{b,ns}	42.67 $\pm$ 2.50 ^b
Total cholesterol (mg/dL)	64.75 $\pm$ 0.55	76.25 $\pm$ 0.83 ^a	66.15 $\pm$ 0.34 ^{b,c}	62.62 $\pm$ 0.92 ^{b,c}	74 $\pm$ 3.57 ^{ns}
Triglycerides (mg/dL)	29.10 $\pm$ 1.07	46.40 $\pm$ 1.33 ^a	25.58 $\pm$ 0.91 ^{b,c}	39.23 $\pm$ 0.78 ^{b,c}	45 $\pm$ 3.74 ^{ns}
VLDL (mg/dL)	5.82 $\pm$ 0.21	9.28 $\pm$ 0.26 ^a	5.12 $\pm$ 0.18 ^{b,c}	7.85 $\pm$ 0.15 ^{b,c}	9 $\pm$ 0.74 ^{ns}
LDL (mg/dL)	6.66 $\pm$ 0.77	27.44 $\pm$ 2.45 ^a	11.35 $\pm$ 1.02 ^{b,c}	9.59 $\pm$ 0.72 ^{b,c}	22.33 $\pm$ 5.17 ^b
Atherogenic index	0.24 $\pm$ 0.01	0.93 $\pm$ 0.09 ^a	0.25 $\pm$ 0.02 ^{b,c}	0.39 $\pm$ 0.02 ^{b,c}	0.74 $\pm$ 0.16 ^b

^a - $P<0.01$: Diabetic control compared with normal control group; ^b - $P<0.01$: MMFR 100, 200 mg/kg and Glibenclamide (5 mg/kg) group compared with diabetic control group; ^c - $P<0.01$: MMFR 100, 200 mg/kg compared with Glibenclamide (5 mg/kg) treated group; ns-non significant; All the values were expressed as mean $\pm$ SD and analyzed for ANOVA and post hoc Tukey test. Difference between groups was considered significant at $P<0.05$ levels

Fig. 2 Effect of MMFR on HbA1c, Hemoglobin and Insulin level in STZ-induced diabetic rats. a- $P<0.01$: Diabetic control compared with normal control group; b- $P<0.01$: MMFR 100, 200 mg/kg and Glibenclamide (5 mg/kg) group compared with diabetic control group; e- $P<0.01$: MMFR 100, 200 mg/kg compared with Glibenclamide (5 mg/kg) treated group; ns-non significant; All the values were expressed as mean $\pm$ SD and analyzed for ANOVA and post hoc Tukey test. Difference between groups was considered significant at $P<0.05$ levels

control group to 44.9 % 8.1 % and 35.6 %, respectively was observed. MMFR at the dose of 200 mg/kg bw also decreased elevated levels of urea, creatinine and uric acid to 28.6 %, 43.2 % and 75.1 %, respectively, which were comparable to that of glibenclamide treated group (40.27 %, 16.2 % and 31.2 %, respectively). Increase in BUN value in diabetic control group (58.18) reduced significantly ($P<0.01$) to 32.03 in MMFR treated group, which was comparable to that of glibenclamide treated group. Total protein level also showed significant increase in MMFR treatment groups (22.6 % and 11.5 %, respectively) but the standard drug treatment groups showed non-significant difference when compared to diabetic control groups (19 %). The increase in urea, creatinine, BUN and uric acid level in diabetic rats were reduced significantly ($P<0.01$) on treatment with MMFR, strengthens the anti-diabetic activity of MMFR and its protective effect on kidney.

Effect of MMFR on antioxidant parameters in diabetic rats kidney

On estimation of kidney homogenate for antioxidant parameters, a significant ($P<0.01$) decrease in the level of GPx (32.6 %), GSH (13.2 %), catalase (5.5 %) and SOD (26.9 %) was observed in diabetic control rats and significant ($P<0.01$) increase in the level of TBARS (22.6 %) was observed in diabetic rats, when compared to normal rats. However on treatment with MMFR at the dose of 100 and 200 mg/kg bw and glibenclamide at the dose of 5 mg/kg bw showed significant ($P<0.01$) increase in the level of GPx (17.9 %, 3.1 %, 53.1 %, respectively), GSH (30.2 %, 2.3 %, 22.4 %, respectively), catalase (5.8 %, 14.3 %, 38.2 %, respectively) and SOD (14.8 %, 0.8 %, 29.6 %, respectively) were observed and significant ($P<0.01$) decrease in the level of TBARS (30.8 %, 30.1 %, 56.25 %, respectively) was observed, when compared to diabetic rats. MMFR 100 mg/kg bw showed

Table 4 Effect of MMFR on urea, creatinine, total protein and uric acid in STZ-induced diabetic rats

Biochemical parameters	Normal control	Diabetic control	MMFR 100 mg/kg bw	MMFR 200 mg/kg bw	Glibenclamide 5 mg/kg bw
SGPT (U/L)	58.83 $\pm$ 1.47	164.0 $\pm$ 1.41 ^a	93.67 $\pm$ 1.63 ^{b,e}	115.5 $\pm$ 1.04 ^{b,e}	58.17 $\pm$ 2.63 ^b
SGOT (U/L)	150.0 $\pm$ 1.41	255.7 $\pm$ 1.86 ^a	188.2 $\pm$ 1.47 ^{b,e}	218.5 $\pm$ 1.87 ^{b,e}	158.2 $\pm$ 4.3 ^b
Urea (mg/dL)	38.37 $\pm$ 3.2	124.6 $\pm$ 2.45 ^a	68.6 $\pm$ 1.38 ^{b,e}	91.2 $\pm$ 1.79 ^{b,e}	74.42 $\pm$ 3.32 ^b
Creatinine (mg/dL)	0.37 $\pm$ 0.05	0.55 $\pm$ 0.05 ^{ns}	0.40 $\pm$ 0.06 ^{ns}	0.53 $\pm$ 0.08 ^{b,ns}	0.43 $\pm$ 0.10 ^b
Total protein (mg)	7.817 $\pm$ 0.17	6.32 $\pm$ 0.07 ^a	7.75 $\pm$ 0.28 ^{b,e}	7.05 $\pm$ 0.18 ^{b,e}	6.11 $\pm$ 0.74 ^{ns}
Uric acid (mg/dL)	1.57 $\pm$ 0.37	3.22 $\pm$ 0.20 ^a	2.13 $\pm$ 0.05 ^{b,ns}	2.75 $\pm$ 0.10 ^{b,e}	2.06 $\pm$ 0.19 ^b
BUN	17.91 $\pm$ 1.31	58.18 $\pm$ 1.00 ^a	32.03 $\pm$ 0.6 ^{b,e}	42.5 $\pm$ 0.7 ^{b,e}	34.75 $\pm$ 1.36 ^b

^a - $P<0.01$: Diabetic control compared with normal control group; ^b - $P<0.01$: MMFR 100, 200 mg/kg and Glibenclamide (5 mg/kg) group compared with diabetic control group; ^c - $P<0.01$: MMFR 100, 200 mg/kg compared with Glibenclamide (5 mg/kg) treated group; ns-non significant; All the values were expressed as mean $\pm$ SD and analyzed for ANOVA and post hoc Tukey test. Difference between groups was considered significant at $P<0.05$ levels

comparable result as that of standard, while MMFR at 200 mg/kg bw group showed antioxidant activity at lesser level (Table 5).

Discussion

MMFR was produced by fermentation of citrinin free mutant *Monascus purpureus* 254 with Indian rice variety IR-532-E-576, and was used for the evaluation of anti-diabetic and nephroprotective potential in diabetic animal model. HPLC analysis of MMFR showed the presence of monacolin K and absence of citrinin (data not shown). As reported in our previous study the anti-diabetic activity may be due to the presence of monacolin K in MMFR (Rajasekaran et al. 2009). In this study food safety of MMFR was evaluated by evaluating its genotoxicity. European food safety authority (EFSA) (EFSA 2008), United states food and drug administration (USFDA) (USFDA 2007) and World health organization (WHO) (WHO 1985) recommends suitable in vitro model for the assessment of genetic toxicity of genetically modified food, as a substitute for animal testing. Thus various functional foods such as genetically modified papaya plant (Gow-Chin Yen et al. 2011), *Antrodia cinnamomea* (Ming-Fang Wu et al. 2011), *Mitragyna speciosa* (Ghazali et al. 2011) etc. reported for the use of Ames test as a suitable test for the evaluation of genotoxicity or mutagenic effect.

In this study, the safety of MMFR was assessed for genotoxicity using Ames bacterial mutagenicity assay as per OECD guidelines (OECD 1997). Ames test was widely used and accepted assay method to detect mutagenic activity of various drugs and chemicals for human use. To assess the genetic toxicity of MMFR three histidine dependant *S. typhimurium* such as TA98, TA100 and TA102 to detect mutation due to frame shift mutation, base-pair substitution and transitional mutation, respectively were used. The results of the test showed MMFR does not produce any mutation with or without metabolic activation system.

To assess the complete safety of MMFR in vivo experiment such as 14 day oral acute toxicity study was performed upto the dose of 5,000 mg/kg bw in female Wister rat. The oral acute toxicity study revealed no sign of toxicity such as death or any abnormality in experimental animals and the LD₅₀ was >5,000 mg/kg bw, which showed the safety of MMFR upto the dose of 5,000 mg/kg bw. Therefore, biological evaluation was carried out using 100 and 200 mg/kg bw dose levels.

The anti-diabetic potential of MMFR was assessed by using STZ-induced diabetic animal models. On treatment with MMFR at the dose of 100 and 200 mg/kg bw showed significant ($P<0.01$) increase in body weight up to 16 % and 22.4 % respectively, which is significantly ($P<0.01$) better than the groups treated with standard anti-diabetic drug glibenclamide at the dose of 5 mg/kg bw it showed 6.5 % increase in body weight. The loss of body weight was observed in STZ-induced diabetic rats may be due to defect in glucose metabolism and the degradation of structural protein (Veeramani et al. 2007). The administration of MMFR improves body weight, when compared to diabetic rats, indicates the preventive effect of MMFR on degradation of structural proteins. Three weeks daily treatment of MMFR showed reduction in blood glucose level. The results showed that MMFR has significant ($P<0.01$) anti-diabetic activity in STZ-induced diabetic rats.

The most common abnormalities in diabetes are change in lipid profile leading to hypertriglyceridemia and hypercholesterolemia. Enhanced serum LDL, VLDL and significant ($P<0.01$) decrease in HDL concentration with increase in atherogenic index was also evident in diabetic control group of the present study. High cholesterol level with increase in atherogenic index is an indicator of cardiovascular diseases (Massinf et al. 2001). The altered lipid profile in diabetic rats were restored to normal level on treatment with MMFR, decrease in cholesterol level ($P<0.01$) and increase in HDL level ($P<0.01$) indicated that MMFR is effective in prevention of atherosclerosis and ischemic heart diseases.

Table 5 Effect of MMFR on antioxidant parameters of STZ-induced diabetic rats in kidney

Antioxidant parameters	Normal control	Diabetic control	MMFR 100 mg/kg bw	MMFR 200 mg/kg bw	Glibenclamide 5 mg/kg bw
GPx (μg of GSH utilized/min/mg of protein)	6.62±0.07	3.46±0.04 ^a	5.265±0.07 ^{b,ns}	4.60±0.08 ^{b,c}	5.3±0.10 ^b
CAT (μM of H ₂ O ₂ /min/mg of protein)	14.55±0.04	10.77±0.07 ^a	14.49±0.05 ^{b,c}	15.74±0.03 ^{b,c}	14.88±0.26 ^b
GSH (μg of GSH consumed/mg of protein)	175.3±1.03	152.0±2.09 ^a	196.2±1.16 ^{b,c}	155.5±0.83 ^{ns,c}	186±3.40 ^b
SOD (μg/mg of protein)	85.51±0.14	62.52±0.11 ^a	71.75±0.75 ^{b,c}	63.0±1.41 ^{ns,c}	81.0±2.36 ^b
LPO (μM of MDA released/min/mg of protein)	2.92±0.11	06.4±0.22 ^a	02.52±0.07 ^{b,c}	2.50±0.08 ^{b,c}	02.8±0.14 ^b

^a $P<0.01$: Diabetic control compared with normal control group; ^b $P<0.01$: MMFR 100, 200 mg/kg and Glibenclamide (5 mg/kg) group compared with diabetic control group; ^c $P<0.01$: MMFR 100, 200 mg/kg compared with Glibenclamide (5 mg/kg) treated group; *ns*-non significant; All the values were expressed as mean ± SD and analyzed for ANOVA and post hoc Tukey test. Difference between groups was considered significant at $P<0.05$ levels

STZ produces type 1 diabetes in experimental animal model by its cytotoxic destruction of pancreatic islet's beta cells (Kumar et al. 2006). The destruction of beta cells of pancreatic islets leads to reduction in insulin level, in turn alters glucose metabolism (Sezik et al. 2005). The anti-diabetic compounds act through mechanism such as improving insulin sensitivity, augmenting glucose- dependant insulin secretion and stimulating regeneration of pancreatic islets cells. To understand the mechanism(s) by which MMFR elicits hypoglycemic activity, various biochemical parameters were evaluated following 3 weeks treatment in rats. The experimental findings showed significant ($P<0.01$) reduction in (i) blood glucose levels on different days, (ii) HbA_{1c} fraction level, (iii) total cholesterol and triglycerides level in both MMFR treated and glibenclamide treated rats suggested the constant control of blood glucose at its normal level was maintained throughout the treatment period. Hemoglobin in red blood cells (RBC) carries oxygen throughout the body, in persistent hyperglycemia glucose in blood combines with hemoglobin and forms glycosylated hemoglobin. HbA_{1c} concentration is an indicator of blood glucose concentration over the period of time and was suggested as an important diagnostic tool for diabetes (McCance et al. 1994). In the present study HbA_{1c} level was increased in diabetic control rats, which was reversed on treatment with MMFR, showed the persistent control of blood glucose level during the entire period of study.

MMFR significantly ($P<0.01$) increases the reduced serum insulin and total protein in diabetic rats. The increase in serum insulin level from beta cells of islets of langerhan's suggested that MMFR regenerates destructed cells of pancreas due to STZ. The studies also suggested that MMFR not only exerts hypoglycemic activity but also have anti-diabetic activity. Increase in transaminase activity of liver in diabetes was reported in many studies (Sumana Ghosh and Suryawanshi 2001); the present study also reported increase in SGOT and SGPT level in diabetic control group, which was reduced on treatment with MMFR showed the hepatoprotective effect of MMFR (Deepak et al. 2007). The increase in urea, creatinine, BUN and uric acid level in diabetic rats were reduced significantly ($P<0.01$) on treatment with MMFR, strengthens the anti-diabetic activity of MMFR and its protective effect on kidney. The level of biochemical parameters in MMFR treated group and control group were in the range as suggested by Charles River for control rats (Mary and Charles 2008), also showed the protective effect of MMFR on kidney of diabetic rat models.

Oxidative stress due to generation of free radicals in diabetes plays a major role in its pathogenesis and its related complications (Laight et al. 2000). The major antioxidant enzymes such as GPx, catalase, and SOD plays first line defense against free radicals generated in vivo during oxidative stress (Yao et al. 2005). The kidneys are the important

organ in the body and plays major role in maintaining normal function of the body. These are the primary organs affected morphologically, functionally and biochemically during persistent diabetes (Vijay 1999). Decrease in oxidative stress markers such as SOD, catalase and GPx were observed in kidneys of diabetic control group, confirms the oxidative stress in kidneys due to STZ-induced diabetes in rat models.

LPO is a free radical mediated process leads to oxidative deterioration of polyunsaturated lipid, under normal physiological conditions low concentration of lipid peroxide was observed in plasma and tissue. Tissue damage due to persistent hyperglycemia mediated by free radicals damages cell membrane through LPO. The level of LPO and reactive oxygen species are common markers of oxidative stress in diabetic rats (Selvan et al. 2008), the same was observed in our study on STZ-induced diabetic rats. The increase in level of kidney TBARS was reversed on administration of MMFR at the dose of 100 and 200 mg/kg bw and glibenclamide at the dose of 5 mg/kg bw, significantly ($P<0.01$). GPx decomposes hydrogen peroxide through oxidation of GSH in normal physiological conditions. The decreased level of GSH and GPx in diabetic rats were reversed on administration of MMFR significantly ($P<0.01$). Catalase is enzyme catalyses the reduction of hydrogen peroxide and protects tissue from damage due to hydroxyl radicals (Chance et al. 1952). The level of catalase was increased significantly ($P<0.01$) on administration of MMFR when compared to diabetic control rats in kidney. The SOD dismutates superoxide anion to hydrogen peroxide and oxygen (Freeman and Crapo 1982). In diabetic rats, kidney SOD level was reduced significantly ($P<0.01$), when compared to normal rats and on treatment with MMFR a significant ($P<0.01$) increase was observed.

The results of this study clearly demonstrated that the MMFR exhibited significant anti-diabetic and protective effect in STZ-induced diabetes and diabetic induced oxidative stress in kidney. Nephroprotective effect of MMFR was due to presence of monacolin K (Rysz et al. 2009). Normal function of kidney in MMFR treatment groups also confirmed the absence of toxicity due to citrinin, a mycotoxin present in traditionally produced MFR. Food safety of genetically modified food MMFR was also confirmed for its absence of genotoxicity and acute toxicity. Thus the regular consumption of MMFR as functional food might be useful in management of diabetes and diabetic induced nephropathy without any side effects and toxic effects.

Acknowledgments Authors acknowledge KMCH College of Pharmacy for providing laboratory facilities to complete the research work described in this article. Authors also thankful to Mr. Ramachandran, Assistant Professor, KMCH College of Pharmacy for providing technical support.

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