Effect of Resveratrol, Curcumin and Metformin on Streptozotocin-Induced Diabetic Nephropathy in Rats

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Abstract: Background: Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli as a complication of diabetes. It is the leading cause of chronic renal failure in the industrialised world. The mechanisms of DN are incompletely understood but include glycosylation of circulating and intrarenal proteins, hypertension and abnormal intrarenal hemodynamics. Resveratrol is a natural polyphenolic compound found in grapes and red wine that has been shown to offer protective effects against many cardiovascular, neurodegenerative diseases and cancer. Curcumin is a natural phenol that may be useful for prevention and treatment of several diseases. Metformin is the first-line drug of choice for treatment of type 2 diabetes, in particular, in overweight and obese people. **Objective:** To study the effect of each of resveratrol, curcumin and metformin on STZ-induced DN in rats. **Methods:** Fifty albino rats were divided into 5 equal groups: Control untreated group, STZ group, STZ + Metformin group, STZ + Resveratrol group and STZ + Curcumin group. Kidney weight/body weight ratio, serum fasting glucose, blood urea, serum creatinine, UAER and creatinine clearance were determined. A part of the kidney was homogenized for determination of tissue TNF-α, TGF-β1, NO, GSH and AGEs and the other part was examined histopathologically. **Results:** Administration of each of resveratrol, curcumin and metformin induced significant increase in creatinine clearance and tissue GSH with significant decrease in kidney weight/body weight ratio, serum fasting glucose, blood urea, serum creatinine, UAER, tissue TNF-α, TGF-β1, NO and AGEs and alleviated the histopathological changes compared to the group that received STZ alone. **Conclusion:** The present study demonstrated the protective effect of each of resveratrol, curcumin and metformin on STZ-induced DN in rats.
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Key words: resveratrol, curcumin, metformin, nephropathy, rats.
Abbreviations: DN (Diabetic nephropathy); STZ (Streptozotocin); UAER (Urinary albumin excretion rate); TNF-α (Tumor necrosis factor alpha); TGF-β1 (Transforming growth factor beta 1); NO (Nitric oxide); GSH (Reduced glutathione); AGEs (advanced glycation end products).

1. Introduction

Diabetic nephropathy (DN) is one of the most frequent life threatening complications of diabetes mellitus that occur in approximately 30-40% of patients. It is usually attributed to metabolic consequences of abnormal glucose regulation, such as elevated blood and tissue levels of glycosylated proteins and heamodynamic changes within the kidney tissue **(Choi *et al.,* 2010)**. The exact mechanisms underlying DN are complex and not well defined. Chronic hyperglycemia, the main determinant of the initiation and propagation of DN, not only generates more reactive oxygen metabolites, but also attenuates anti-oxidative mechanisms through non-enzymatic glycation of the scavenging enzymes. Moreover, hyperglycemia may be toxic either by non-enzymatic reaction of glucose with proteins and subsequent formation of advanced glycosylation end products (AGEs) or by increased metabolism leading to increased oxidative stress and activation of protein kinase C, resulting in increased production of proinflammatory cytokines **(Valko *et al.,* 2007)**.

Resveratrol is a natural polyphenol found in grapes and red wine. It has been shown to offer protective effects against many cardiovascular and neurodegenerative diseases and cancer**.** Although the mechanisms of action of resveratrol have not yet been clearly elucidated, many studies have reported this to its antioxidant, anti-inflammatory and anti-apoptotic effects **(Palsamy & Subramanian, 2009; Chang *et al.,* 2011).**

Curcumin, a yellow pigment from *Curcuma longa*, is a major component of turmeric that has been used as a pain relieving anti-inflammatory agent to relieve pain and inflammation in the skin and muscles. It served as a treatment for jaundice, menstrual difficulties, hematuria, hemorrhage, colic and flatulence **(Sinha *et al.,* 2003)**. Research has focused on curcumin's antioxidant, anti-inflammatory, anti-carcinogenic and anti-microbial properties and on its use in cardiovascular diseases and prevention of kidney injury and restoring kidney function **(Sharma *et al.,* 2007)**.

Metformin is an antidiabetic agent that decreases intestinal absorption of glucose, increases

its anaerobic metabolism, improves insulin sensitivity and decreases glucagon release. Recent investigations strongly showed that metformin prevented oxidative stress-induced death in several cell types, including endothelial cells through a mechanism dependent on the mitochondrial permeability transition pore opening **(El-Mir *et al.*, 2008; Morales *et al.*, 2010).** Also, the ability of metformin to modulate several oxidative stress markers and proinflammatory cytokines at the biochemical and gene expression levels may play a role in its renoprotective effect **(Alhaider *et al.*, 2011).**

The aim of this study is to investigate the effect of each of resveratrol, curcumin and metformin on streptozotocin (STZ)-induced DN in rats.

2. Materials and Methods

Chemicals and drugs:

- Streptozotocin (STZ), metformin, resveratrol and curcumin were purchased from Sigma Chemical Co (St. Louis, MO, USA). STZ was dissolved in 0.1 M cold citrate buffer (pH 4.5). Metformin was dissolved in normal saline. Resveratrol and curcumin were suspended in 0.5% carboxymethyl cellulose solution.
- Commercially available kits, Diamond diagnostics, for estimation of blood glucose, blood urea and serum creatinine.
- ELISA kits supplied by RayBiotech, Inc. for determination of TNF-α.
- ELISA kits supplied by Wuhan EIAab Science Co.,Ltd for determination of advanced glycation end products (AGEs).
- ELISA kits supplied by Uscn Life Science Inc. Wuhan, for determination of transforming growth factor beta 1 (TGF-β1).

The present study was carried out on 50 albino rats weighing 150-200 grams collected from local source with free access to food and tap water *ad libitum* through the whole period of the study. All the experiments were conducted according to the National Research Council's guidelines. Animal handling was followed according to Helsenki declaration of animal ethics. Rats were divided into five equal groups each of 10 rats as follows:

Group I: Control untreated group.

Group II: Received a single intraperitoneal injection of STZ in a dose of 65 mg/kg b.wt. **(Alhaider *et al.*, 2011)**.

Group III: Received a single intraperitoneal injection of STZ (65 mg/kg b.wt.) then received metformin orally in a dose of 500 mg/kg b.wt./day **(Alhaider *et al.*, 2011)**.

Group IV: Received a single intraperitoneal injection of STZ (65 mg/kg b.wt.) then received resveratrol orally in a dose of 5 mg/kg b.wt./day **(Palsamy & Subramanian, 2009).**

Group V: Received a single intraperitoneal injection of STZ (65 mg/kg b.wt) then received curcumin orally in a dose of 60 mg/kg/day **(Sharma *et al.*, 2007)**.

One week after STZ injection blood samples were collected from retro-orbital sinus of rats and were used for estimation of serum glucose level. Rats with glucose level above 300 mg/dL were considered diabetic. Drugs were administered one week after STZ injection and continued for 8 weeks.

At the end of the work, rats were kept in special metabolic cages for 24 hours urine collection. Urine samples were centrifuged at 1400 rpm for 5 minutes after proper dilution and the supernatant was collected to determine 24 hours urinary protein levels using a colorimetric method described by **Faas *et al.* (2010)**. The urinary albumin excretion rate (UAER) was measured using the following formula according to **Alhaider *et al.* (2011)**:

UAER (mg/24 hrs) = 24 hrs total volume of urine (L) X Urinary protein levels (mg/L)

After an overnight fasting, blood samples were taken from retro orbital sinus of rats. Blood samples were kept in glass tubes in a water bath for 30 minutes at 37°C till blood clotting occurred. Then, serum was separated by centrifugation for 20 minutes to estimate fasting serum glucose level according to the method of **Trinder (1969)**, blood urea according to the method of **Patton & Crouch (1977)** and serum creatinine according to the method of **Henry (1974)**. According to the 24 hours urine volume, urinary creatinine and serum creatinine concentration, creatinine clearance was calculated by applying the following formula according to **Cockroft & Gault (1976)**:

$$\text{Creatinine clearance (ml/min)} = \frac{\text{mg creatinine/dl urine X ml urine 24 hours}}{\text{mg creatinine /dl serum X 1440}}$$

Rats were sacrificed by decerebration and both kidneys were removed, weighed for determination of kidney weight/body weight ratio and sectioned for histopathological analysis **(Hammersen, 1985)**. The remaining kidney tissue was homogenized for determination of tissue TNF-α using ELISA kits, tissue nitric oxide (NO) (Tissue nitrite and nitrate were estimated as an index of NO production) **(Cortas & Wakid, 1990)**, tissue TGF-β1 using ELISA kits, advanced glycation end products (AGEs) using ELISA kits **(Makita *et al.*, 1992)**, reduced glutathione (GSH) according to the method of **Beutler *et al.* (1963)** and tissue total protein content according to the method of **Lowry *et al.* (1951)**.

Statistical analysis:

Results are expressed as the mean ± SEM. Comparison between different groups was carried out by one-way analysis of variance test (ANOVA) followed by LSD test. The statistical significance was accepted at a level of $P < 0.05$.

3.Results

Effect of different treatments on serum fasting glucose:

Administration of STZ to rats induced significant increase in serum fasting glucose compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in serum fasting glucose compared to STZ-treated group (Table 1).

Effect of different treatments on kidney weight/body weight ratio:

Administration of STZ to rats induced significant increase in kidney weight/body weight ratio compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in kidney weight/body weight ratio compared to STZ-treated group (Table 2).

Effect of different treatments on UAER:

Administration of STZ to rats induced significant increase in UAER compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in UAER compared to STZ-treated group (Table 1).

Effect of different treatments on blood urea:

Administration of STZ to rats induced significant increase in blood urea compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in blood urea compared to STZ-treated group (Table 1).

Effect of different treatments on serum creatinine:

Administration of STZ to rats induced significant increase in serum creatinine compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in serum creatinine compared to STZ-treated group (Table 1).

Effect of different treatments on creatinine clearance:

Administration of STZ to rats induced significant decrease in creatinine clearance compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant increase in creatinine clearance compared to STZ-treated group (Table 1).

Effect of different treatments on tissue TNF-α:

Administration of STZ to rats induced significant increase in tissue TNF-α compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in tissue TNF-α compared to STZ-treated group (Table 2).

Effect of different treatments on tissue TGF-β1:

Administration of STZ to rats induced significant increase in TGF-β1 compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in TGF-β1 compared to STZ-treated group (Table 2).

Effect of different treatments on tissue NO:

Administration of STZ to rats induced significant increase in tissue NO compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in tissue NO compared to STZ-treated group (Table 2).

Effect of different treatments on tissue advanced glycation end products (AGEs):

Administration of STZ to rats induced significant increase in tissue AGEs compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant decrease in tissue AGEs compared to STZ-treated group (Table 2).

Effect of different treatments on tissue GSH:

Administration of STZ to rats induced significant decrease in tissue GSH compared to the normal control group. Administration of metformin, resveratrol or curcumin induced significant increase in tissue GSH compared to STZ-treated group (Table 2).

Table (1): The effect of different treatments on blood urea, serum creatinine, creatinine clearance, serum fasting glucose and UAER in the studied groups

Group / Parameter	Group I Control	Group II STZ	Group III STZ+ Metformin	Group IV STZ+ Resveratrol	Group V STZ+ Curcumin
Blood urea mg/dl	20.2 ± 0.67	77.12± 2.83*	32.41 ± 2.77 #	41.64 ±2.23#	37.4 ± 2.15#
Serum creatinine mg/dl	0.25 ± 0.03	2.23 ± 0.04*	0.79 ± 0.03#	1.17 ± 0.03#	1.07 ± 0.02#
Creatinine clearance (ml/min)	1.24 ± 0.02	0.34 ± 0.03*	0.98 ± 0.04#	0.79 ± 0.05#	0.87 ± 0.05#
Serum fasting glucose mg/dl	119.11± 2.31	464.6 ±10.9*	147.42 ±3.07#	176.35 ± 3.06#	207.22 ±3.25 #
UAER mg/day	2.81±0.21	11.92±0.43*	4.91 ±0.15#	5.8±0.32#	6.61±0.4#

Number of rats in each group = 10; Values expressed as mean ± SEM.

* Significant compared to control group; # Significant compared to STZ group

Table (2): The effect of different treatments on tissue TNF-α, TGF-β_1, NO, AGEs, GSH and kidney weight/body weight ratio in the studied groups.

Group / Parameter	Group I Control	Group II STZ	Group III STZ+ Metformin	Group IV STZ+ Resveratrol	Group V STZ+ Curcumin
Tissue TNF-α pg/mg protein	82.33±15.55	225.63±220.6*	119.63±12.41#	121.34±15.4#	138.12±17.12#
Tissue TGF-β_1 pg/µg protein	3.3±0.11	10.44±0.2*	4.94±0.31#	5.61±0.21#	6.12±0.1#
Tissue NO (µmol/g protein)	1.34 ± 0.12	3.96 ± 0.08*	1.75 ± 0.09#	1.97 ± 0.13#	2.11 ± 0.06#
Tissue AGEs (U/mg protein)	3.18±0.11	7.87±0.33*	4.22±0.22#	5.15±0.21#	4.85±0.13#
Tissue GSH (µmol/g tissue)	4.3±0.04	2.03±0.07*	3.81±0.06#	3.41±0.09#	3.12±0.08#
kidney weight/body weight ratio	6.04 ± 0.2	18.91± 0.88*	9.62±0.5#	10.71 ± 0.51#	12.13± 0.82#

Number of rats in each group = 10 Values expressed as mean ± SEM.
* Significant compared to control group # Significant compared to STZ group

Histopathological results:

- In the control group, the histopathological examination of kidney tissue showed, normal appearance of the glomeruli & tubules (Fig. 1).
- Administration of STZ resulted in severe glomerulosclerosis and cellular infiltration, interstitial fibrosis, tubular dilatation, casts and atrophy together with vascular arteriosclerosis (Fig. 2).
- Treatment with either metformin, resveratrol or curcumin resulted in marked decrease in glomerulosclerosis, cellular infiltration and interstitial fibrosis with mild dilatation of the tubules (Fig. 3, Fig. 4, Fig.5).

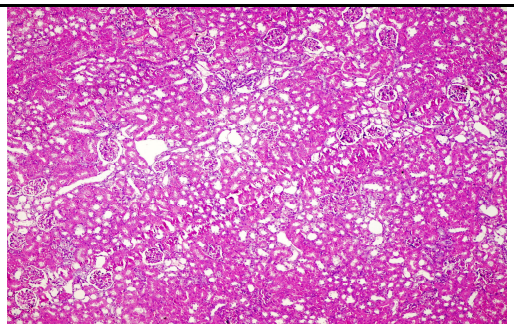

Fig. 1: H&E stained sections from the kidney of control untreated group with normal appearance of the glomeruli, tubules and interstitium

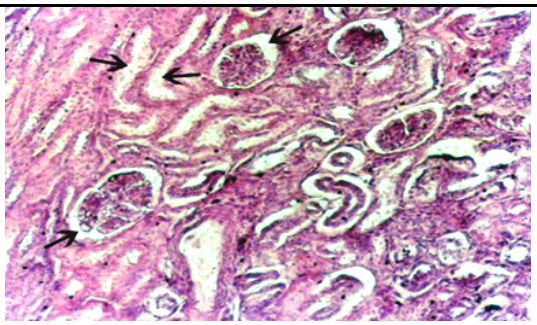

Fig. 3: H&E stained sections from the kidney of STZ+ Metformin treated group with apparently normal glomeruli, mild interstitial fibrosis and minimal tubular necrosis

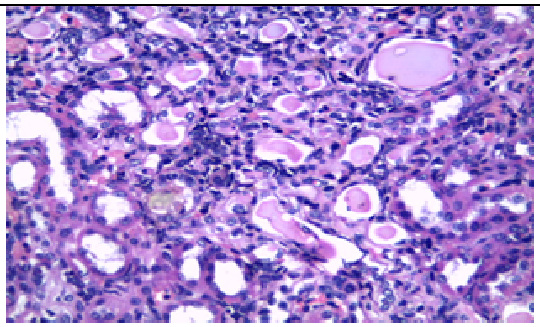

Fig. 2: H&E stained sections from the kidney of STZ treated group showed glomerulosclerosis, interstitial fibrosis, tubular dilatation, casts & atrophy

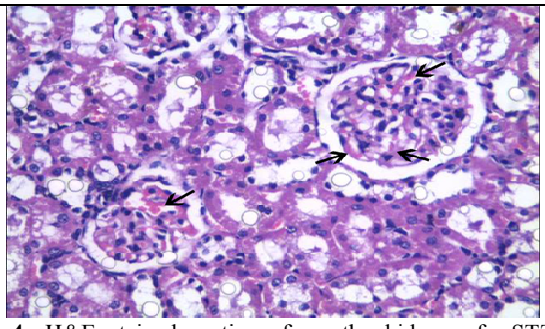

Fig. 4: H&E stained sections from the kidney of STZ + Resveratrol treated group with marked decrease in glomerulosclerosis, cellular infiltration and interstitial fibrosis with mild dilatation of the tubules.

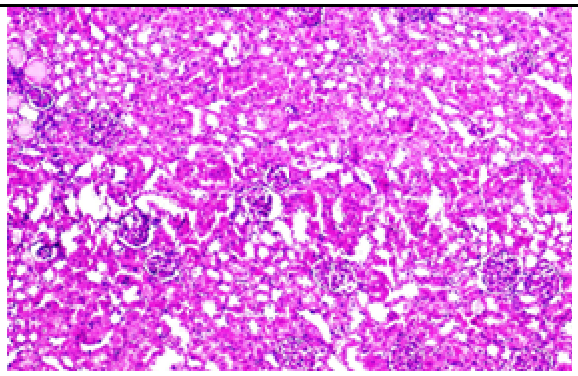

Fig. 5: H&E stained sections from the kidney of STZ+Curcumin treated group with minimal glomerulosclerosis, minimal cellula infiltration and mild dilatation of the tubules.

4. Discussion

Diabetic nephropathy is the most common cause of end-stage renal disease and is one of the leading causes of morbidity and mortality in patients with diabetes. It is initially characterized by glomerular haemodynamic abnormalities that lead to glomerular hyperfiltration which overtime progress to glomerular structural damage evidenced by microalbuminuria, proteinuria, decreased glomerular filtration rate and end-stage renal failure (Soldatos & Cooper, 2008). DN occurs as a result of the deleterious effects of both metabolic and hemodynamic insults, which at the cellular level lead to the activation of intracellular signaling pathways and transcription factors, thus triggering the production and release of cytokines, and growth factors such as TGF-β, increased NO production and accumulation of AGEs which mediate and/or amplify the renal damage. This ultimately leads to the structural and functional features characteristic of DN (David & Peter, 2008). In the present study, the administration of STZ to rats induced a model of DN manifested as an increase in serum fasting glucose, blood urea, serum creatinine, UAER, tissue TNF-α, tissue TGF-β, tissue AGEs, tissue NO and kidney weight/body weight ratio with significant decrease in creatinine clearance and tissue GSH with marked histopathological changes manifested by massive glomerulosclerosis, interstitial fibrosis, tubular dilatation, casts and atrophy compared to the normal control group. These results are in agreement with other studies that indicated that exposure to STZ causes biochemical and histopathological features of DN (Palsamy & Subramanian, 2009; Choi *et al.,* 2010; Alhaider *et al.,*2011, Chen *et al.,*2011).

Tesch & Allen (2007) reported that STZ can selectively damage the insulin-producing β-cells in the pancreas resulting in hyperglycaemia. This hyperglycemia is regarded as one of the leading causes in the progression of DN. Accumulating evidence also suggested that the development of DN was associated with the activation of several stress-sensitive signal pathways, including nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) which lead to overexpression of the genes encoding growth factors such as TGF-β. Additionally, it was reported that both oxidative stress and proinflammatory cytokines accelerated the pathological process of DN (Chang *et al.,*2011). Also, AGEs stimulate mesangial cells and increase production of extracellular matrix proteins such as fibronectin, enhance production of reactive oxygen species and NO, stimulate intracellular molecules such as NF-κB, MAPK and cytokines which play an important role in the progression of DN (Soldatos & Cooper, 2008; Chen *et al.,*2011).

Metformin is an oral anti-diabetic drug that is used, along with other drugs, in treatment of type II diabetes mellitus. Recent studies showed that metformin has antioxidant and anti-inflammatory properties which may play a role in the protection against DN (Alhaider *et al.,* 2011; Takiyama *et al.,*2011) which is in agreement with the results of the present study which showed that administration of metformin resulted in decrease in serum fasting glucose, blood urea, serum creatinine, UAER, tissue TNF-α, tissue TGF-β, tissue AGE, tissue NO and kidney weight/body weight ratio with significant increase in creatinine clearance and tissue GSH with improvement of the histopathological picture compared to STZ-treated group.

The therapeutic action of metformin is attributed mainly to its effects on the hepatocytes, with decreased glucose production and on the muscle cells, with enhanced glucose uptake (Cheng *et al.,* 2006). Recently, Chakraborty *et al.,* (2010) have demonstrated that treatment of type II diabetic patients with metformin restores the antioxidant status, enzymatic activity and inflammatory parameters, and hence improves the status of oxidative and nitrosative stress altered in DM. Alhaider *et al.,* (2011) reported that metformin

attenuates DN by modulation of oxidative stress markers at the gene expression level, with the consequent improvement in mitochondrial function and energy production and blocks the induction of proinflammatory cytokines such as TNF-α and IL-6 genes. Moreover, metformin can affect the carbonyl stress which prevents AGEs formation and improves the free radical defense system (Faure *et al.,*2008). Also, metformin was found to decrease the expression of growth factors such as TGF-β which plays an important role in the pathogenesis of DN (Viollet *et al.,*2012).

Resveratrol is a polyphenolic phytoalexin that occurs naturally in many plant species, including grapes and berries and exhibits many pharmacologic health benefits including antioxidant, antimutagenic, anti-inflammatory, antiplatelet, anticancer and cardioprotective properties (Jiang *et al.,*2012). There has been epidemiologic and clinical evidence confirming that resveratrol may act as an antioxidant through enhancing hydrogen peroxide tolerance and inhibiting cyclooxygenase-2 activity. Recently, resveratrol has been reported to possess antihyperglycemic effect in experimental diabetes, which is mediated by modulating the activities of key carbohydrate metabolizing enzymes in the hepatic and renal tissues of experimental diabetic rats (Su *et al.,*2006; Palsamy & Subramanian, 2009). These studies are in accordance with the results of the present study where administration of resveratrol resulted in decrease in serum fasting glucose, blood urea, serum creatinine, UAER, tissue TNF-α, tissue TGF-β, tissue AGE, tissue NO and kidney weight/body weight ratio with significant increase in creatinine clearance and tissue GSH with improvement of the histopathological picture compared to STZ-treated group.

Chang *et al.* (2011) reported that resveratrol retards progression of DN through modulation of oxidative stress, proinflammatory cytokines and AMP-activated protein kinase. Kitada *et al.* (2011) found that resveratrol improves oxidative stress and protects against DN through normalization of Mn-superoxide dismutase dysfunction and restoration of tissue GSH level. Also, resveratrol was found to suppress the augmented TGF-β/smad signalling in STZ-induced diabetic rats (Chen *et al.,*2011). Moreover, resveratrol was demonstrated to affect oxidative stress markers such as malondialdehyde and GSH and antioxidant enzymes such as superoxide dismutase and catalase in the renal tissues of diabetic rats (Sharma *et al.,*2006). Resveratrol was reported to alleviate diabetic vasculopathy through attenuation of AGE-receptor for AGE signalling pathway, inhibition of AGE-induced mesangial cell proliferation which represents an important factor in the pathogenesis of DN and arresting cells in the S phase of the cell cycle (Jiang *et al.,*2012).

Curcumin is a naturally occurring yellow pigment isolated from the plant Curcuma longa and is a potent antioxidant, free radical scavenger and anti-inflammatory agent (Fujisawa *et al.,*2004). Along with being an inhibitor of lipid peroxidation, curcumin also is an inhibitor of nitric oxide synthase (NOS) overexpression and of nuclear factor kappa B activation (*et al.,*2006). In the present study, the administration of curcumin resulted in significant decrease in serum fasting glucose, blood urea, serum creatinine, UAER, tissue TNF-α, tissue TGF-β, tissue AGE, tissue NO and kidney weight/body weight ratio with significant increase in creatinine clearance and tissue GSH with improvement of the histopathological picture compared to STZ-treated group which was in agreement with Sajithlal *et al.* (1998), Sharma *et al.* (2006) and Soetikno *et al.* (2011).

The efficacy of curcumin has been widely observed in reducing various diabetic secondary complications such as DN and reduction of AGEs (Sajithlal *et al.,* 1998; *et al.,* 2006). The hypoglycemic effect of curcumin was attributed to its ability to inhibit glucose output and/or stimulate insulin secretion from the pancreas (Kim *et al.,*2007). Curcumin was found to increase the antioxidant status of pancreatic B-cells and inhibit the activation of both TNF-α and NF-kB which have an important role in the development of DN (Weisberg *et al.,*2008). Also, Soetikno *et al.* (2011) reported that curcumin attenuated the expression of TGF-β1, osteopontin, p300 and extracellular matrix proteins such as fibronectin and type IV collagen and inhibits nitric oxide synthetase activity. Moreover, curcumin was found to reduce the level of AGEs and the cross-linking of collagen in diabetic rats. The oxidative stress in diabetic rats was observed to reduce significantly on curcumin administration. Similarly, the accumulation of lipid peroxidation products in serum of diabetic rats was reduced significantly by curcumin. The preventive effect of curcumin on the advanced glycation and cross-linking of collagen was more pronounced than its therapeutic effect (Jain *et al.,*2006).

In conclusion, the present study demonstrated the protective effect of each of resveratrol, curcumin and metformin on STZ-induced DN in rats due to their anti-inflammatory and antioxidant properties together with their inhibitory effects on AGEs and TGF-β in renal tissues.

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