Research Article

EFFECT OF *CITRULLUS COLOCYNTHIS* IN AMELIORATE THE OXIDATIVE STRESS AND NEPHROPATHY IN DIABETIC EXPERIMENTAL RATS

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ABSTRACT

Diabetic nephropathy reflects certain microvascular complications of diabetes mellitus that are including diabetic nephropathy, neuropathy and retinopathy. Nephropathy first becomes manifest with hyperfiltration and microalbuminuria. Present work aimed to illustrate the influence of *Citrullus colocynthis* fruits extract in protection of the diabetic kidney functions and tissues.

Materials and Methods: Thirty male albino rats were divided into three groups (ten in each). Group I (Normal group) was intraperitoneal (I.P) injection with a vehicle and served as control group. Group II was I.P injection with freshly prepared streptozotocin (STZ) 30mg/kg body weight for three consecutive days and served as diabetic control group. Group III was I.P injection with STZ and then received extract of *Citrullus colocynthis* fruit (50mg/kg/day) orally for 50 days and served as diabetic treated group. Specimens from the kidneys were collected and fixed in 10% buffered neutral formalin solution and then investigated histopathologically.

Results: Collected data showed a significant decrease in blood glucose, urea, creatinine, microalbuminuria and uric acid while GSH, GPx and SOD demonstrated significantly increased. While, the activities of ALP, AST and γ -GT in the kidney tissues of diabetic rats while the activity of ALT was not altered. The histopathological findings were coincided with our biochemical findings in both diabetic and treated groups. Diabetic kidney shows atrophy of renal curpusle, shrinkage of capillary within increase Bowman's space while, diabetic rat received *Citrullus colocynthis* fruit extract showed partial protection of glumeruli and appeared nearly normal.

Conclusion: The present study clearly demonstrated that the medicinal plant *Citrullus colocynthis* fruit may have protective effects on the kidney functions and tissues. So it may play a role in prevent nephropathy as one of microvascular complications of diabetes mellitus.

KEY WORDS: Hyperfiltration, Nephropathy, Kidney, Microalbuminuria, Fruits

INTRODUCTION

Diabetes Mellitus (DM) is a common endocrine disease, possibly the world's fastest growing metabolic disease. DM is possibly the world's largest growing metabolic disease and as the knowledge on the heterogenecity of this disorder is advanced, the need for more appropriate therapy increases (David and Granner 1996). Diabetic nephropathy reflects certain microvascular complications of diabetes mellitus (Krolewski et al., 1985, Klein et al., 1988). Hyperfiltration and microalbuminuria were the first signs of diabetic nephropathy, so the glycemic control is the main means for possible prevention or modification of the natural history of diabetic microvascular complications (Verrotti et al., 1999). Nephropathy is a serious microvascular complication of diabetes mellitus which is preceded by a period of microalbuminuria. Increased loss of proteoglycan from glomerular basement membrane has been postulated to alter glomerular charge selectivity which contributes to urinary loss of albumin (Mc-Auliffe et al., 1996). Microalbuminuria is a term refered to urinary albumin excretion (Mangili 1998), where the poor glycaemic control correlates directly with hyperfiltration and renal hyperfusion in diabetics prognosis (Soper et al., 1998). A comprehensive array of endogenous scavengers has been identified. They were either enzymatic (SOD, catalase and selenium- and non-selenium-dependent GPx) or non-ezymatic (vitamins A, E and C, GSH, bilirubin, albumin, selenium, uric acid and coenzyme Q-10). They exist in both the aqueous and membrane compartments of the cells (Cotgreave, et al., 1988; Krinsky, 1992). The endogenous scavengers are equipped to deal with the normal low level production of free radicals during normal metabolic activity and to limit their damaging effects (El-Khatib, 1997). The level of O^{-2} (superoxide anion) is mainly controlled by both spontaneous and enzymatic dismutation by SOD (Fridovich, 1989; 1995). Two separate types of SOD enzymes were present in hepatocytes. Cytosolic SOD contains copper and zinc as its active site while mitochonderial SOD contains manganese as its active

site (Fridovich, 1989; 1995). Human SOD levels show great tissue heterogeneity, they are high in liver and certain brain areas, but low in erythrocytes and lung tissue (Cotgreave, et al., 1988). Subsequent studies have identified SOD as enzyme system that appears to have specifically evolved to deal with ($O_2^{\bullet-}$) as a substrate and provides the second layer of defense after GSH-Px and catalase against free radical injury. These enzymes catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 (Sciuto, 1998). Provinviali, et al., 2002 reported that, Superoxide dismutase has been cloned and the recombinant human enzyme has been expressed in yeast (Fridovich, 1975). Recently, it has been found that both recombinant superoxide dismutase and catalase prolong the survival of amyotrophic lateral sclerosis (ALS) mice models after disease onset (Fubini, and Hubbard, 2003). The use of human antioxidant enzymes, obtained by genetechnology, may permit the treatment of a variety of clinical conditions associated with oxidative stress (Tainer, et al., 1983 & Fubini and Hubbard, 2003). Oxidative stress plays an important role in the chronic complications of IDDM where, hyperglycemia is involved in the generation of oxygen-free radicals (Lee et al., 2002). Biological antioxidants are compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidations (Krinsky 1992). They could also be referred to as scavengers (Salvemini and Botting 1993). *Citrullus colocynthis* contained large amounts of phenolics and flavonoids that have antioxidant activities (Kumar et al., 2008). *Citrullus colocynthis* had a beneficial effect on improving the glycemic profile without severe adverse effects in type II diabetic patients (Huseini et al., 2009). It was used as purgative, anthelmentic, antipyritic, carmanative, cures tumours, leucoderma, asthma, jaundice, enlargment of spleen, tuberculous glands of the neck, elephantiases and ulcers, also reported that fresh fruit and seeds are eaten as an laxative and removing kidney stones (Bolous, L., 1983; Shah et al., 1989). The Present work was designed mainly to investigate biochemical effects of *C-colocynthis* fruit extract on diabetic nephropathy and oxidative stress in experimental diabetic rats.

MATERIALS AND METHODS

Experimental Animals

Thirty male albino rats (200–220 g) were selected for the present study. Rats were obtained from the National Research Centre Cairo, Egypt. These rats were fed with basic diet containing barley and carrots and allowed to free access of tap water and kept under constant environmental conditions at room temperature.

Preparation of *Citrullus colocynthis* fruit extract:

The fruit pulp were crushed, soaked overnight for 2 weeks in a suitable amount of 70 % ethyl alcohol and filtered through Wattman filter paper no.1. The obtained filterate was evaporated under vacum using *Heidolph rotatory evaporator, Germany,* till exhaustion. The extract for plant was weighted, labeled and kept in refrigerator at 4°C till use in further experiment (Shan and Khan,1997).

Induction of Diabetes

This done by intraperitoneal (I.P) injection of streptozotocin (STZ), purchased from Sigma-Aldrich Chemie (Deisenhofen, Germany), dissolved in citric acid buffer (pH 4.2) at a dose level 30mg/kg body weight for three consecutive days and injected within 10 min of dissolution (Povoski et al.,1993). Blood glucose was determined and rats with a blood glucose level more than 180 mg/dl were selected for the study. Animals were divided into three groups (ten in each). Group I (Normal animals) was I.P injection with a vehicle and served as normal group. Group II was I.P injection with STZ [diabetic rats] and served as diabetic control group. Group III was I.P injection with STZ and then received extract of *Citrullus colocynthis* fruit (50mg/kg/day) orally for 50 days (Abdel-Hassan and Abdel-Barry 2000) and served as diabetic treated group.

Blood Collection and Biochemical Analysis

After 50 days, the rats were fasted over night, collected blood in coated glass tubes (Schemer 1967) via retro–orbital bleeding for each animal and each sample was collected into 2 tubes, heparinized and non-heparinized. The non heparinized blood samples were allowed to coagulate and then centrifuged at

3000 x*g* for 15 min at 4°C. The separated sera used for the estimation of serum level of glucose, urea, creatinine and uric acid. The heparinized blood samples were divided into 2 aliquots. The first aliquot was used for determination of glutathione peroxidase (GPx) activity according to (Kumar et al., 2006). The second aliquot was haemolyzed using bidistilled water and the haemolysate of each sample was divided into two portions were treated with chloroform/ethanol (3:5 V/V) mixture to precipitate and the resultant supernatant was used for the determination of Superoxide Dismutase (SOD) activity according to Liu et al., (2007). The second portion was deproteinized with meta-phosphoric acid and the clear supernatant was used for the estimation of GSH level by Öktem (2006). Glucose was determined spectrophotometrically as described by Trinder (1969). Urea was determined spectrophotometrically as described by Potton and Crouch, (1977), Creatinine was estimated in serum according to method of (Seeling and Wust 1969) and serum uric acid was estimated according to method of (Fossati, 1980).

Preparation of kidney homogenate

The kidneys were then quickly removed, washed in ice-cold, isotonic saline and blotted individually on ash-free filter paper. The tissues were then homogenized in 0.1 M Tris-HCl buffer, pH 7.4 at 4°C. The crude tissue homogenate was then centrifuged at a speed of 9000 rpm for 15 min at room temperature and the supernatant was kept at -20°C for biochemical analysis. The homogenate used for the estimations of tissue enzymes activities.

The enzymes: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) were assayed according to Reitman and Frankel (1957) and Alkaline phosphatase (ALP) was assayed according to Kind and King 1954 and the enzyme γ-glutamyl transpeptidase (γ-GT) was assayed by the method of Rosalki and Rau, (1972).

Determination of microalbuminuria

Urine Sample

Morning urine samples collected individually and stored at 4 ˚C protected from light (Gonzalez et al., 1999) and processed later for microalbuminuria (MA) determination. Total protein urine was determined according to Watanabe et al., (1986) using SENTINEL diagnostic kits (MILAN- ITALY).

Histological study

Kidneys were collected and fixed in 10% buffered neutral formalin solution and then investigated histopathologically.

Tissue characterization procedure

Kidneys tissues were stained with heamatoxylin and eosin for tissue characterization and organ identification. Histological specimens were examined under the light microscope.

Statistical analysis

Collected data were analyzed by one-way ANOVA utilizing computerized statistical program (InStat).

RESULT AND DISCUSSION

Results

Tables (1 and 2):

The STZ diabetic rats demonstrated significant increase in blood glucose, urea, creatinine, uric acid and microalbuminuria while GSH, GPx and SOD demonstrated significant decrease (P<0.001) as compared to normal group. Oral administration of *Citrullus colocynthis* fruit extract for 50 days for diabetic rats resulted in significant decrease in blood glucose, urea, creatinine, uric acid and microalbuminuria while GSH, GPx and SOD demonstrated significant increase (P < 0.001) as compared to diabetic control group (Tables 1 and 2).

Table 3:

Table 3 showed the activities of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP) and Gamma Glutamyl Transferase (γ-GT) in kidney. In the kidney of non-treated diabetic rats, the activities of ALP, AST and γ-GT were increased, while the activities of ALT was not altered. Oral administration of *Citrullus colocynthis* for 50 days resulted in the decrease of the activities of ALP, AST and γ-GT in the kidney of diabetic rats while the activities of ALT was not altered.

Table 1: Effects of orally repeated doses of *Citrullus colocynthis* fruits extract on serum glucose, urea, creatinine, uric acid and microalbumin in urine of diabetic rats for 50 days.

Parameter	Normal	Diabetic Control	Diabetic treated
Glucose (mg/dl)	82.8 ± 2.15	198.1 ± 3. 51 *	93.9 ± 2.79 **
Urea (mg/dl)	15.35 ± 0.7	28.8 ± 1.06 *	19.0 ± 0.68 **
Creatinine (mg/dl)	0.52 ± 0.03	2.4 ± 0.14 *	1.0 ± 0.05 **
Uric acid (mg/dl)	5.35 ± 0.36	11.5 ± 0.44*	6.94 ± 0.39**
MA (µg/min)	48.1 ± 2.57	237.9 ± 9.5 *	71.4 ± 2.84 **

Values were mean ± SE.

*Significantly different from normal control at P < 0.001.

**Significantly different from diabetic control at P < 0.001.

Table 2: Effects of orally repeated doses of Citrullus colocynthis fruits extract on blood glutathione (GSH) levels, blood glutathione peroxidase (GPx) and superoxid dismutase (SOD) activities of diabetic rats for 50 days.

Parameter	Normal	Diabetic Control	Diabetic treated
GSH (mg/g Hb)	41.2 ± 0.47	15.9 ± 0.56 *	42.5 ± 1.28 **
GPx (U/g Hb)	146.6 ± 4.07	56.2 ± 5.03 *	158.3 ± 5.09 **
SOD (U/g Hb)	15.45 ± 1.44	7.2 ± 0.42 *	18.9 ± 1.22 **

Values were mean ± SE.

*Significantly different from normal control at P < 0.001.

**Significantly different from diabetic control at P < 0.001.

Table 3: Effects of orally repeated doses of Citrullus colocynthis fruits extract on activities of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phospahtase (ALP) and γ-glutamyl transferase (γ GT) in kidney tissues of diabetic rats for 50 days.

Parameter	Normal	Diabetic Control	Diabetic treated
AST (IU/gm)	520.0 ±13.0	880.5 ±14.2*	575.0 ±10.0**
ALT (IU/gm)	660.4 ±1580	654.2 ±21.1	632.6 ±13.1
ALP (IU/gm)	0.17±0.01	0.54±0.04*	0.28±0.02**
γ GT (IU/gm)	2.32±0.20	5.27±0.46*	3.28±0.14**

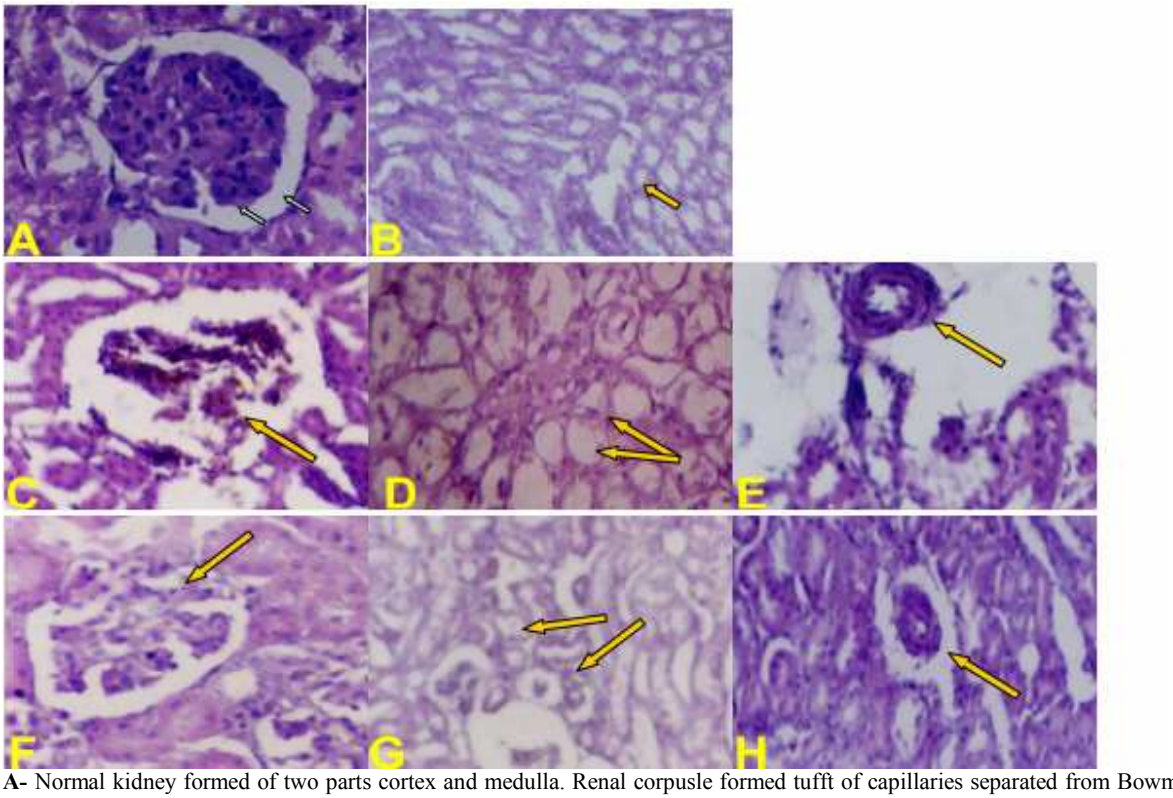
Values were mean ± SE.

*Significantly different from normal control at P < 0.001.

**Significantly different from diabetic control at P < 0.001.

Histopathological Findings:

The kidneys of STZ diabetic rats revealed renal corpusle atrophy with shrinkage of capillary, damage of glomeruli and increased Bowman's space (fig. C). Renal tubule appeared pale, vaculated, edematous, dilated with acidophilic hyaline material (fig. D) joined with dilated and congested blood vessels (fig. E). Oral administration of *Citrullus colocynthis* fruit extract showed partial protection of glumeruli and appeared nearly normal with wide tubules and many vaculated cells. Pale dilated renal tubule with acidophilic hyaline material and thickning of blood vessels were observed (fig. F, G, H).



A- Normal kidney formed of two parts cortex and medulla. Renal corpusle formed tufft of capillaries separated from Bowman's capsule by Bowman's space *(Haematoxylin and eosin x 400).*

B- Normal renal tubule appeared rounded and lined by cuboidal epithelium *(Haematoxylin and eosin x 400).*

C- Diabetic kidney shows atrophy of renal curpusle, shrinkage of capillary within increase Bowman's space *(Haematoxylin and eosin x 400)***.**

D- Diabetic kidney shows pale, vaculated edematous, dilated renal tubule with acidophilic hyaline material *(Haematoxylin and eosin x 400).*

E- Blood vessels of diabetic kidney shows dilated and congested blood vessels *(Haematoxylin and eosin x 400).*

F- Diabetic rat received *Citrullus colocynthis* fruit extract showed, partial protection of glumeruli and appeared nearly normal *(Haematoxylin and eosin x 400).*

G- Diabetic rat received *Citrullus colocynthis* fruit extract showed, wide tubules and vaculated cells *(Haematoxylin and eosin x 400).*

H- Diabetic rat received *Citrullus colocynthis* fruit extract showed, many vaculated, pale dilated renal tubule with acidophilic hyaline material and thickning of blood vessels *(Haematoxylin and eosin x 400)*.

DISCUSSION

Diabetes is recognized as one of the leading causes of morbidity and mortality in the world; about 2.5-7% of the world's population diagnosed with diabetes mellitus (Seghrouchni et al., 2002). Despite of the effect of anti-hyperglycemic drugs and insulin sensitizers, there remains side effects that necessitate finding other alternatives. Medicinal plants provide such valuable therapeutic alternative (Reaven *et al*. 1983). More than 400 traditional plant treatments for Diabetes Mellitus (DM) have been reported, but only a small number of these have received scientific and medical evaluation (Bailey and Day, 1989; Ivorra et al. 1989). Diabetes Mellitus (DM) is a chronic disease characterized by high blood glucose levels due to an absolute or relative deficiency of circulating insulin levels. Although various types of oral hypoglycemic agent are currently available along with insulin for treating DM, there were a growing interest in herbal remedies due to the side-effects associated with the existing therapeutic hypoglycemic agents (Kameswara and Appa 2001).

Diabetes mellitus also causes renal damage due to abnormal glucose regulation, including elevated glucose and glycosylated protein tissue levels, haemodynamic changes within the kidney tissue and increased oxidative stress (Aurell and Bjorck 1992). The present results demonstrated that blood urea, uric acid and creatinine levels were higher in non-treated diabetic rats than in control group (Table 1). The

level of these substances had reduced after treatment by *Citrullus colocynthis*, which may indicate the ability of this extract to enhance renal functions. These results are in agreement with other previous studies (Saeed et al 1995; Mohammad Dallak et al, 2009). Serum uric acid was reduced in rats treated with ethanolic extract of *Citrullus colocynthis* fruit. In addition, an anti-inflammatory action of *Citrullus colocynthis* was estemated by (Badawi, et al., 1998; Callejas, et al., 1998). Treatment with *Citrullus colocynthis* fruit extract had beneficial effect as a complimentary therapy on improving glycemic profile in type II diabetic patients (Al-Ghaithi et al., 2004; Falah Hosseini et al., 2006). *Citrullus colocynthis* extract possesses A hypoglycemic effect of this extract acts through an increase in insulin production and the subsequent increase in activity of glycolytic enzyme and decrease in activity of enzymes of gluconeogenesis (Mohammad Dallak et al,2009). *Citrullus colocynthis* had antihyperglycemic as well as insulinotropic actions in diabetic rats (Mahmoud Al-Khateeb et al., 2009). The antidiabetic action of *Citrullus colocynthis* was probably due to enhanced insulin secretion or due to increase in preipheral glucose uptake, decreases gluconeogensis and inhibited release of counter-regulatory hormones (Qixuan, et al., 2002). On the other hand, it has been suggested that the mechanism responsible for the serum glucose lowering effect of *Citrullus colocynthis* were attributed to an inhibitory effect of glucose absorption (Meir and Yaniv, 1985), an increased incorporation of circulating glucose as hepatic glycogen (Welihinda and Karuna-nayake, 1986), or an enchanced secretion of insulin (Welihinda,et al., 1986; Higashino, et al., 1992). Multiple mechanisms have been proposed as the cause of *Citrullus colocynthis's* hypoglycemic properties. Components of *Citrullus colocynthis* extract appear to have structural similarities to animal insulin, as measured by electrophoresis and infrared spectrum analysis (Lee, et al., 2000). In transaminase reaction, one amino acid converted to the corresponding keto acids with simultaneous conversion of another keto acid to an amino acid. The aminotransferases (enzymes) involved in this reaction are: Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) (Ganong 2001). In the present study, AST activities of these enzymes in kidneys was increased but ALT activities was not altered in non treated diabetic rats than in control group (Table 3). The enzyme Alkaline Phosphatase (ALP) is present in the canalicular and sinusoidal membrane of many tissues. In the present study activity of this enzyme raised in kidney of non-treated diabetic rats compared to control group (Table 3), this increases was indicated by others (Kumar and Clark, 2005). Fifty days of treatment with *Citrullus colocynthis* led to a reduction in the activity of this enzyme, a result that could support our earlier deduction that this plant extract may have the ability to protect kidney damage. γ-glutamyl transpeptidase is a microsomal enzyme that is present in kidney and liver tissues (Hada et al., 1978). γ-GT rises in parallel with ALP as it has a similar of excretion. The increase in γ-GT seen in non-treated diabetic rats has also reduced after treatment by *Citrullus colocynthis*, indicating, again, the healing ability of this plant extract on tissues of kidney. γ-glutamyl transpeptidase activity was found to be increased in kidneys due to oxidative stress, where the GSH level was only slightly decreased (Juan Cutrín et al., 2000).

Oxidative stress is a key factor in many human diseases (Rice and Gopinathan, 1995; Toyokuni, 1999). Reactive oxygen species (ROS) have the potential to damage nucleic acids, proteins and biomembranes. When cellular defense mechanisms fail, severe dysfunction or cell death can result, events that are part of the respective pathogenic process. There is accumulating evidence that plant derived antioxidants may reduce or prevent oxidative stress and have a beneficial influence on animal and human health (Gebhardt, 2002). Present result demonstrated that fruit extract of *Citrullus colocynthis* was effective in enhancing the GSH levels as well as GPx and SOD activities. This was in agreement with the results of other studies (Haider, 1996 & Zhao and Agarwal, 1999). Dhanasekar and Sorimuthu, 2005, reported that, oral administration of *Citrullus colocynthis* extracts induced an increase in reduced glutathione, superoxide dismutase and glutathione peroxidase (Raza, et al., 1996).

Microalbuminuria is known to be a harbinger of serious complications in type 2 diabetes mellitus (Chirag Parikh et al 2004). There was a direct

correlation of microalbuminuria with duration of diabetes. Prevalence of microalbuminuria is around 37% in type-2 diabetes mellitus. Incidence of microalbuminuria increases with age as well as with increased duration of diabetes mellitus (Chowta et al., 2009). Control of diabetes with regular treatment also play a role in the development of diabetic nephropathy (Jungmann et al., 2001; Mogensen et al., 2000; Levin et al., 2000).

Structural changes in diabetic kidney may be evident in spite of absence of microalbuminuria. Thickening of basal membrane and increasing mesangial matrix volume were examples of these changes, and attributed mostly to long standing of hyperfiltration and poor metabolic control (Berg et al.,1998).

The present study indicated that STZ diabetic rats (control group) demonstrated renal corpusle atrophy with shrinkage of capillary and increased Bowman's space. Renal tubule appeared pale, vaculated, edematous, dilated with acidophilic hyaline material joined with dilated and congested blood vessels. Damage or loss of glomeruli in diabetes could lead to such an increase albumin in urine (fig. C, D, E). Oral administration of *Citrullus colocynthis* fruit extract showed partial protection of glumeruli and appeared nearly normal with wide tubules and many vaculated cells. Pale dilated renal tubule with acidophilic hyaline material and thickning of blood vessels were observed (fig. F, G, H).

CONCLUSION

The present study clearly demonstrated that the medicinal plant *Citrullus colocynthis* fruit extract may have the ability to enhance kidney functions and tissues. So it play a role in prevent nephropathy as one of microvascular complications of diabetes mellitus.

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