Journal of Anatomical Sciences

Email:anatomicaljournal@gmail.com

J Anat Sci 11 (2)

Hibiscus Sabdariffa Reduces Renal Damage in Experimental Streptozotocin-Induced Diabetes

Adeyemi DO and Adewole OS
Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, College of Health Science, Obafemi Awolowo University, Ile-Ife, Nigeria.

Corresponding author: David Olawale Adeyemi
E-mail: waledavade@yahoo.com; +2348028101028

ABSTRACT
This study evaluated the nephroprotective and antioxidant potential of methanolic extract of *Hibiscus sabdariffa* L. calyx (HSCE) on normal and streptozotocin-induced diabetic Wistar rats.
Type 1 diabetes mellitus was induced in Wistar rats by a single intraperitoneal injection of 80 mg/kg b.w. Streptozotocin (STZ) dissolved in 0.1 M citrate buffer (pH 6.3). The rats were divided into five groups (n =12) including normal control group, test group I, diabetic negative control, test group II, and diabetic positive control. The test groups received 1.75 g/kg bw of HSCE by gavage for 15 days. Animals were sacrificed; their kidney tissues and serum were evaluated for histopathological and biochemical parameters. The protective effects of the extract on STZ-diabetes induced renal damage was evident from the results of the histopathological analysis and the biochemical parameters evaluated in the serum and kidney homogenates. Reduced levels of glutathione, catalase and superoxide dismutase in the kidney of diabetic rats were significantly improved in the *H. sabdariffa* -treated rats ($p < 0.05$). Elevated concentrations of urea and creatinin in the serum of diabetic rats were also significantly lowered ($p < 0.05$) in HSCE-treated rats. Examination of stained kidney sections revealed hydropic glomerular and tubular degenerations, Bowman space diminution, glomerular and tubular basement membrane thickening as well as excessive deposition of glycogen and collagen in the renal interstitium. The kidneys of HSCE-treated rats were protected from these pathological changes. The protective effects of the extract on STZ-diabetes-induced renal damage could be partly related to its antioxidant activity.

Keywords: Streptozotocin diabetic nephropathy; *Hibiscus sabdariffa*; Antioxidant; *Anthocyanin*; Protocatechuic

INTRODUCTION
Diabetes mellitus (DM) is a group of metabolic diseases characterized with inappropriate hyperglycemia due to either a deficiency of insulin secretion or a combination of insulin resistance and inadequate insulin secretion[1]. Diabetic nephropathy, one of the most serious complications of diabetes has become the leading cause of end-stage renal failure in many countries[2, 3]. At present, diabetic kidney disease affects about 15 to 25% of type 1 diabetic patients[4] and 30 to 40% of patients with type 2 diabetes[5, 6].

Diabetic nephropathy is characterized by specific renal morphological and functional alterations. Features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion (UAE), glomerular basement membrane (GBM) thickening, and mesangial expansion with the accumulation of extracellular matrix (ECM) proteins such as collagen, fibronectin, and laminin. Advanced diabetic nephropathy is characterized by proteinuria, a decline in renal function, decreasing creatinine clearance (CrCl), glomerulosclerosis, and interstitial fibrosis[4, 7].

There are several new approaches to the treatment of diabetic nephropathy based on an ever-growing mechanistic understanding of the causes of diabetic nephropathy by the specific pathogenic roles. These agents include pharmacologic inhibitors of advanced glycation end products (AGEs) formation, protein kinase C (PKC), oxidative stress, and transforming growth factor β (TGF-β)[8].

Oxidative stress is known to play a significant role in the pathogenesis of diabetic complications[9]. High levels of oxidative stress with excessive generation of free radicals, and depleted levels of free radical scavenging enzymes have been demonstrated in several studies, both in experimental animal models of diabetes and in human diabetic subjects[10, 11]. In type 1 diabetes, reactive oxygen species (ROS) are involved in *β*-cell dysfunction initiated by autoimmune reactions and inflammatory cytokines[12]. In type 2 diabetes, ROS activate β cell apoptotic pathways, impair insulin synthesis and also contribute to insulin resistance[13].

There are many evidences suggesting that ROS play an important role in the pathogenesis of diabetic nephropathy[14]. To prevent the development and progression of diabetic nephropathy, it would be

effective in combing the strategies to prevent overproduction of ROS and to increase the removal of preformed ROS[14].

A wide variety of natural products have been found to possess ability to control metabolic problems and oxidative stress in diabetes. Among them, *Hibiscus sabdariffa* L. (*Malvaceae*) is a valuable source of traditional medicine[15]. *Hibiscus sabdariffa* plant is known in many countries and is consumed as hot and cold beverages popularly called "zobo" in Nigeria. It is known as roselle in English, karkade in Arabic and yakuwa, amukan/isapa and okworo ozo in Hausa, Yoruba and Ibo languages of Nigeria respectively.

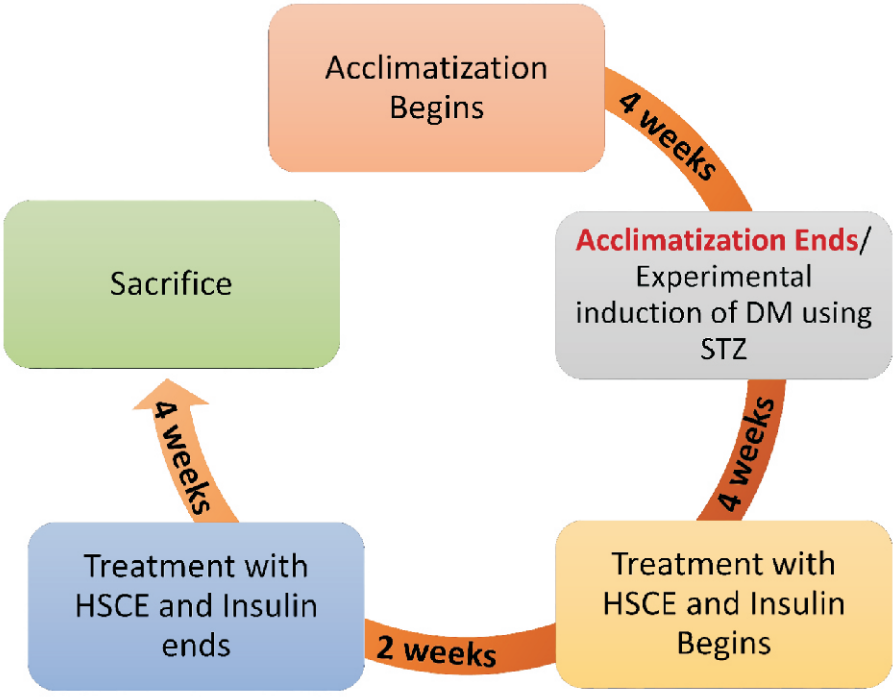
Water-soluble extracts from *Hibiscus sabdariffa* calyx contain several antioxidants, such as polyphenolic acids, flavonoids, protocatechuic acid[16] and anthocyanins[17]. Recent studies have shown the potentials of these antioxidants in the protection of kidney in chemically-induced kidney damage[18, 19]. Hence, this study investigates the potential nephroprotective properties of the polyphenol-rich extract of *Hibiscus sabdariffa* in animal model of STZ-diabetes-induced kidney damage.

MATERIALS AND METHODS

Chemicals: All chemicals used were of analytical grade. STZ was purchased from Sigma-Aldrich (**St. Louis, MO, USA**). Biochemical kits for creatinine and urea assay were purchased from Randox Laboratory (Crumlin, Co. Antrim, UK). Biochemical kits for catalase, GSH, GPx and TBARS assay were purchased from Bio Assay System (Hayward, CA 94545, USA) while the kit for SOD assay was purchased from Cell Technology Inc. (Mountain View, CA 94043). Other histological reagents and stains were purchased from Sigma-Aldrich (St. Louis, MO, USA)

Animals: Sixty healthy Wistar rats (150 – 200 g) of both sexes obtained from the animal holding of the College of Health Sciences, Obafemi Awolowo University Ile-Ife were used for the experiment. They were maintained under standard environmental conditions of temperature, humidity and light and fed on standard rat pellets (Ladokun Feeds, Ibadan, Nigeria) and water *ad libitum*. The animals were acclimatized to the laboratory for four weeks. The rats received humane care according to the criteria stipulated in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Research, National Research Council, USA[20] and their experimental use was approved by the Health Research and Ethics Committee of Obafemi Awolowo University Ile Ife.

Experimental Design: The animals were randomly assigned into five groups A, B, C, D and E of twelve rats each. Group A was normal control (normoglycemic rats); group B was test group I (normoglycemic rats treated with *Hibiscus sabdariffa* calyx extract); group C was diabetic negative control (untreated diabetic rats given STZ as described in "Induction of Experimental Diabetes" section); group D was test group II (diabetic rats treated with **HSCE**); *while* group E was diabetic positive control (diabetic rats treated with protamin zinc insulin). The design of the experiment followed a previously reported scheme shown in Figure 1 [21].



Figue 1: Schematic diagram of the experimental design

Plant Material and Extraction: *Hibiscus sabdariffa* calyx extract was prepared as previously reported[21, 22.] Dried and pulverized calyxes of *H. sabdariffa* (200 g) were extracted three times with 70 % methanol (500 mL × 3) and continuously stirred at room temperature for 24 hours each. The extract was concentrated *in vacuo* at 25 °C using a vacuum rotary evaporator (RE 100B, Bibby Sterilin, United Kingdom) and the aqueous phase was partitioned with ethyl acetate (EtOAc). The aqueous fraction (coded HSCE) was freeze dried using a vacuum freeze drier (FT33-Armfield, England) and used for the experiment.

Phytochemical Analysis: Phytochemical analysis of alkaloids, anthocyanins, flavonoids, phenols, saponins, tannins coumarins, triterpens and steroids was carried out on calyx of *Hibiscus sabdariffa* as earlier reported[22, 23, 24].

Induction of Experimental Diabetes and Drug Administration: Animals were fasted (but still allowed access to water) for 16 hours prior to induction. Diabetes mellitus was induced in groups C, D and E rats by a single intraperitoneal (i.p.) injection of 80 mg/kg bw STZ dissolved in 0.1 M sodium citrate buffer (pH 6.3) as previously reported[22, 25, 26]. Group A rats were injected with equivalent volumes of citrate buffer i.p. Four weeks post induction of diabetes, daily doses of 1.75g/kg bw HSCE was administered orally to the rats in test groups I and II (groups B and D) for 15 days by gavage, while group E rats (diabetic positive control) were treated with 1 IU/kg/day (i.p.) of protamine zinc insulin. Rats in group C (diabetic negative control) were left untreated.

Sacrifice: A mid-line incision was made through the anterior abdominal walls of the rats under terminal chloroform anesthesia. The kidney tissues were excised and weighed. Some of the tissues were fixed in 10 % formol saline for 48 hours for histological procedures. Some others were fixed in Bouin's fixative for 24 hours for histochemical procedure, while other parts were frozen for biochemical assay.

Determination of Kidney Weight: At sacrifice, the absolute weight of the kidneys was measured using a top loader sensitive balance. The relative weight of the kidney (%) was calculated from the body weight at sacrifice and the average of the absolute weights of the two kidneys as earlier described[26].

$$Relative\ weight\ of\ the\ kidney = \frac{Average\ Absolute\ kidney\ weight}{Body\ weight\ at\ sacrifice} x\ 100$$

Histological and Histochemical Procedures: Kidney tissues fixed in 10 % formol saline were processed via paraffin wax embedding method[27]. Sections of 4 µm thickness produced were stained with haematoxylin and eosin (HE) for general histological examination of the kidney tissues and with Masson trichrome stain (MT) to histologically demonstrate collagen fibres in kidney. The tissues fixed in Bouin's fixative were processed via paraffin wax embedding. Sections of 4 µm thickness produced were stained with PAS with diastase control to histochemically demonstrate glomerular and tubular basement membranes in the kidney sections. The sections were examined under Leica DM750 research microscope with a digital camera (Leica ICC50) attached. Digital photomicrographs were taken at various magnifications.

Histomorphometry: All histomorphometric studies were carried out on Leica DM750 research microscope connected to a digital camera (Leica ICC50) and a laptop computer (Acer Aspire One) with Image J 1.42q and open office.org image analysis software installed. The kidney sections were examined at different magnifications and the following parameters were measured: (a) the maximum glomerular diameter, (b) the maximum width of the Bowman space, (c) proximal convoluted tubule (PCT) trans-luminal diameter (d) distal convoluted tubule (DCT) trans-luminal diameter and (e) PCT and DCT epithelial thickness. The trans-luminal diameter of PCT and DCT were derived by measuring the maximum diameter (D_1) and minimum diameter (D_2) at right angle to the maximum diameter. The transluminal diameter (D) and the cross-sectional area (A_c) of the renal tubules (PCT and DCT) were calculated using the following equations:

$$D = \sqrt{D_1 D_2} \qquad Ac = \frac{\pi D^2}{4}$$

where π is equivalent to 3.142

Biochemical Assays-- Collection of Blood Samples: Blood samples were obtained from the rats by cardiac puncture at sacrifice and were kept for 30 minutes at room temperature. Serum was separated from the blood samples by centrifugation at 5000 rpm for 10 min at room temperature.

Assay for Kidney Function Markers: Serum marker of kidney (creatinine and urea) were estimated spectrophotometrically, using enzymatic colorimetric assay kits (Randox, Crumlin, Co. Antrim, UK).

Preparation of Kidney Homogenates: The excised kidney was divided into separate portions for the estimation of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione (GSH) and thiobarbituric reactive substances (TBARS). Homogenates were obtained for each of the assays as previously described[22].

Biochemical Assay for Antioxidants and Lipid Peroxidation Markers: The activities of CAT, SOD and GPX as well as the concentration of GSH and TBARS were determined in the resulting supernatants by a 96-well microplate-based assay using their specific quantitative colorimetric detection kits following manufacturers manual [28, 29, 30, 31, 32].

Statistical Analysis: All values were presented as mean ± standard error of mean (SEM) for twelve rats in each of the five group of rats. The significance of difference in the means of all parameters was determined using one-way analysis of variance (ANOVA; 95% confidence interval). Dunnett multiple comparison (DMC) and Student Newman-Keul's (SNK) *post hoc* tests were carried out for comparison of all groups with control and comparison of all pairs of groups respectively. All statistics were carried out in GraphPad Prism. Values of $p < 0.05$ were considered as significant[33].

RESULTS

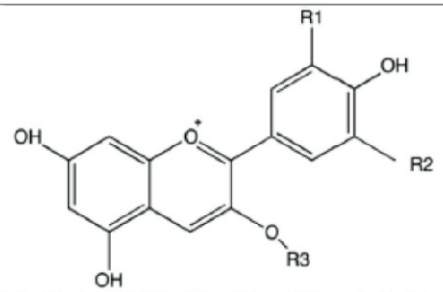
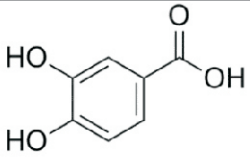
Phytochemical Constituents of *Hibiscus Sabdariffa*: Phytochemical screening of HSCE in this study revealed the following constituents: flavonoids, phenols, tannins, saponins, alkaloids, triterpenes and steroids (Table 1). Anthocyanins (delphinidin-3-sambubioside and cyanidin-3-sambubioside), flavonoids (protocatechuic acid and quercetin-3-rutinoside) and organic acids (hydroxycitric acid and hibiscus acid) had been previously isolated from extracts of *Hibiscus sabdariffa* calyx[34, 35, 36, 37]. The chemical structures of these constituents and their percentage compositions are shown in Table 2 [38].

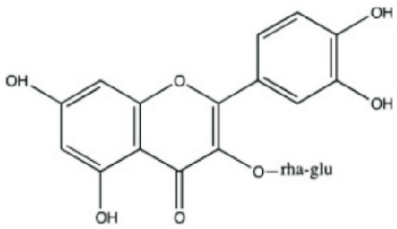
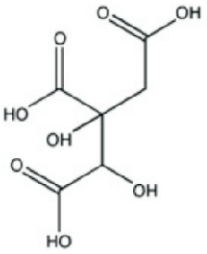
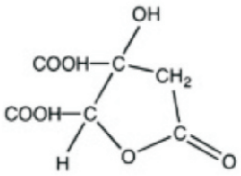
Table 1: Phytochemical Screening of *Hibiscus sabdariffa* Calyx Extract

Class of Compounds	Ethanol Extract
Alkaloids	+
Flavonoids	+++
Saponins	++
Anthocyanins	+++
Tannins	+
Triterpene and steroids	+
Phenols	++
Coumarins	-

Note: (-): Absence; (+): Less presence; (++): Moderate presence; (+++) Abundant presence

Table 2. Chemical structure of isolated compounds from *Hibiscus sabdariffa* calyx

Class	Compound	Chemical structure	% composition
Anthocyanins	Delphinidin-3-sambubioside, Cyanidin-3-sambubioside	 Cyanidin-3-sambubioside (R1= OH; R2= H; R3= Sambubioside) Delphinidin-3-sambubioside (R1= OH; R2= OH; R3= Sambubioside)	
Flavonoids and phenolic acid	Protocatechuic acid		24.24

	Quercetin-3-rutinoside.		0.89
Organic acid			
	Hydroxycitric acid		12 – 20
	Hibiscus acid		13 - 24

Effects of HSCE on Kidney Weight: The effects of HSCE on the body weight, absolute and relative kidney weight in all groups of rats are shown in Table 3. The diabetic positive and negative controls presented with severe loss in body weight and absolute kidney weight when compared with the other groups. However, the relative weight of the kidneys with respect to the body weight was not significantly different in all groups.

Table 3: Effects of *H. Sabdariffa* on body weight and kidney weight

	Body weight (g)	Absolute kidney weight (g)	Relative kidney weight (%)
A (normal control)	181.92 ± 6.49†	0.74 ± 0.06†	0.41 ± 0.03†
B (Test I)	179.75 ± 7.11†	0.72 ± 0.07†	0.40 ± 0.04†
C (Diabetic -ve control)	141.58 ± 3.33 *‡	0.52 ± 0.05*‡	0.37 ± 0.02†
D (Test II)	168.17 ± 6.15†	0.68 ± 0.05†	0.40 ± 0.03†
E (Diabetic +ve control)	139.41 ± 2.55*‡	0.54 ± 0.04*‡	0.39 ± 0.03†

* $p < 0.05$ compared with the normal control, determined by one-way ANOVA followed by DMC *post hoc* test.
† ‡ within column signifies $p < 0.05$ between groups with different symbols, determined by SNK *post hoc test*

Effects of HSCE on Kidney Function Markers: The concentrations of creatinin, urea, total protein and albumin in normoglycemic, diabetic negative and positive controls, and extract treated groups are presented in Table 4. The concentrations of creatinin and urea significantly increased ($p < 0.05$) in the serum of diabetic negative control rats when compared with the normoglycemic rats. Administration of HSCE significantly lowered ($p < 0.05$) the serum level of these kidney function markers in test group II rats better than that demonstrated by protamin zinc insulin administration in the positive control group. HSCE had no significant effect on the serum levels of urea and creatinin in normoglycaemic rats. On the other hand, the levels of total protein and albumin were significantly reduced ($p < 0.05$) in the serum of diabetic negative control rats when compared with the normoglycemic rats. However, the serum levels of total protein and albumin were comparable in the normogycemic and test groups I and II rats.

Table 4: Effects of *H. Sabdariffa* on the kidney function markers

	Creatinin (μmol/L)	Urea (mmol/L)
A (normal control)	77.20 ± 6.15†	3.89 ± 0.94†
B (Test I)	65.02 ± 5.13†	3.35 ± 0.83†
C (Diabetic -ve control)	305.46 ± 11.84*‡	11.87 ± 1.85*‡
D (Test II)	103.32 ± 6.17†	7.83 ± 1.34*§
E (Diabetic +ve control)	283.62 ± 9.79*‡	9.03 ± 1.45*§

* $p < 0.05$ compared with the normal control, determined by one-way ANOVA followed by DMC *post hoc* test.
† ‡ § within column signifies $p < 0.05$ between groups with different symbols, determined by SNK *post hoc test*

Effects of HSCE on Kidney Antioxidants: The activities of the antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) and the concentration of glutathione (a non-enzymatic antioxidant) were significantly reduced ($p < 0.05$) in the kidney homogenates of the diabetic negative control rats compared with normoglycemic rats (table 3). HSCE treatment elevated the activities of these antioxidant enzymes and concentration of glutathione significantly ($p < 0.05$) in the kidney of test group II rats better than that demonstrated by protamin zinc insulin in the diabetic positive control group (Table 5). However, HSCE treatment had no significant effect on these antioxidants in the kidney of normoglycemic rats.

Table 5: Effects of *H. Sabdariffa* on the antioxidants and lipid peroxidation marker in kidney homogenate

	CAT (U/L)	GPx (U/L)	SOD (U/mL)	GLU (μM)	TBARS (μM MDA)
A (normal control)	0.86 ± 0.07†	1.12 ± 0.07†	98.76 ± 5.02†	7.34 ± 0.65†	8.05 ± 0.73†
B (Test I)	0.91 ± 0.05†	1.17 ± 0.07†	94.55 ± 3.11†	7.56 ± 0.71†	9.01 ± 0.55†
C (Diabetic -ve control)	0.47 ± 0.03*‡	0.68 ± 0.05*‡	45.38 ± 2.91*‡	2.08 ± 0.33*‡	16.49 ± 0.96*‡
D (Test II)	0.79 ± 0.04†	0.78 ± 0.06*‡	92.17 ± 4.02†	7.03 ± 0.57†	9.24 ± 0.58†
E (Diabetic +ve control)	0.55 ± 0.03*‡	0.72 ± 0.04*‡	36.79 ± 2.68*§	2.65 ± 0.31*‡	11.73 ± 0.86*§

* $p < 0.05$ compared with the normal control, determined by one-way ANOVA followed by DMC *post hoc* test.
† ‡ § within column signifies $p < 0.05$ between groups with different symbols, determined by SNK *post hoc test*

Effects of HSCE on Lipid Peroxidation: The concentration of TBARS, a marker of lipid peroxidation was significantly higher ($p < 0.05$) in the kidney of diabetic negative control rats than in normoglycemic rats (Table 5). However, HSCE treatment lowered the level of TBARS significantly ($p < 0.05$) in the treated diabetic rats.

Histopathological Assessment of the Kidney: Histological study of the kidney of normoglycaemic rats showed normal glomerulus (G) surrounded by bowman capsule (arrow), proximal convoluted tubules (P) and distal convoluted tubules (D) (Figure 2). Examination of the sections of the kidney of STZ diabetic rats revealed an array of pathological changes including hydropic glomerular and tubular degenerations (H), Bowman space diminution (Figures 2 and 3), accumulation of collagen fibres (Figure 3), glomerular and tubular basement membrane thickening, as well as excessive glycogen deposition (Figure 4). These pathological changes which persisted in diabetic positive control rats treated with protamin zinc insulin were ameliorated in the kidneys of test group II rats treated with HSCE (Figures 2, 3 and 4). However, HSCE had no effects on the kidneys of normoglycaemic rats.

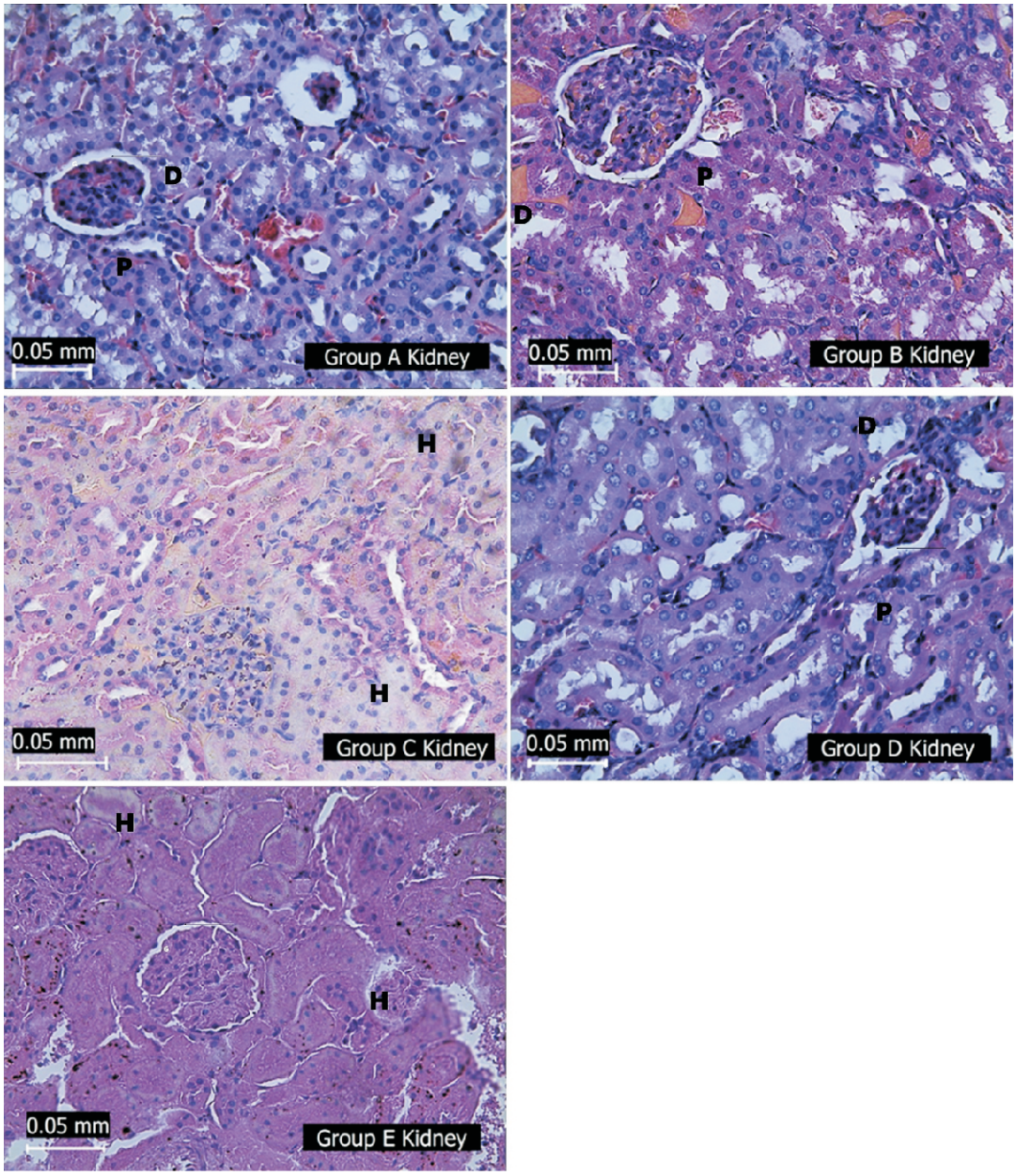


Figure 2: Photomicrographs of H & E stained paraffin section from the cortex of the kidneys of experimental rats (A - normoglycemic rats, B – test group I, C – diabetic negative control, D – test group II and E – diabetic positive control). The glomerulus (G) is surrounded by the Bowman space (arrow), proximal convoluted tubules (P) and distal convoluted tubules (D). Pathological changes including hydropic glomerular and tubular degenerations (H) and Bowman space diminution were observed in the kidneys of groups C and E rats. The kidney sections of groups A, B and D rats appeared normal.

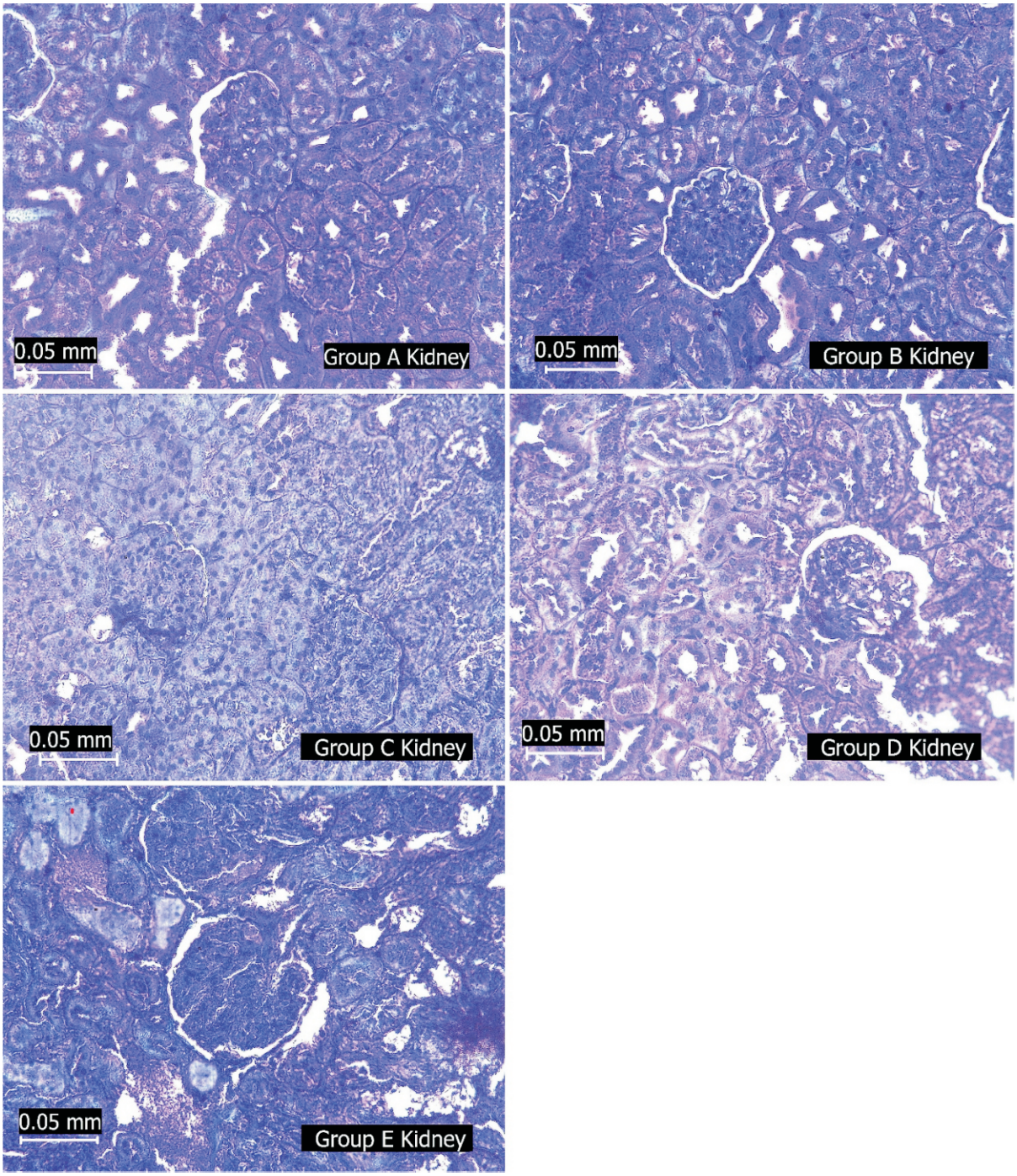


Figure 3: Photomicrographs of Massons' trichrome stained paraffin section from the cortex of the kidneys of the experimental rats (A - normoglycemic rats, B – test group I, C – diabetic negative control, D – test group II and E – diabetic positive control). Extensive area of renal interstitium showed the presence of collagen (light green stained area) in the kidneys of groups C and E rats. The kidney sections of groups A, B and D rats appeared normal.

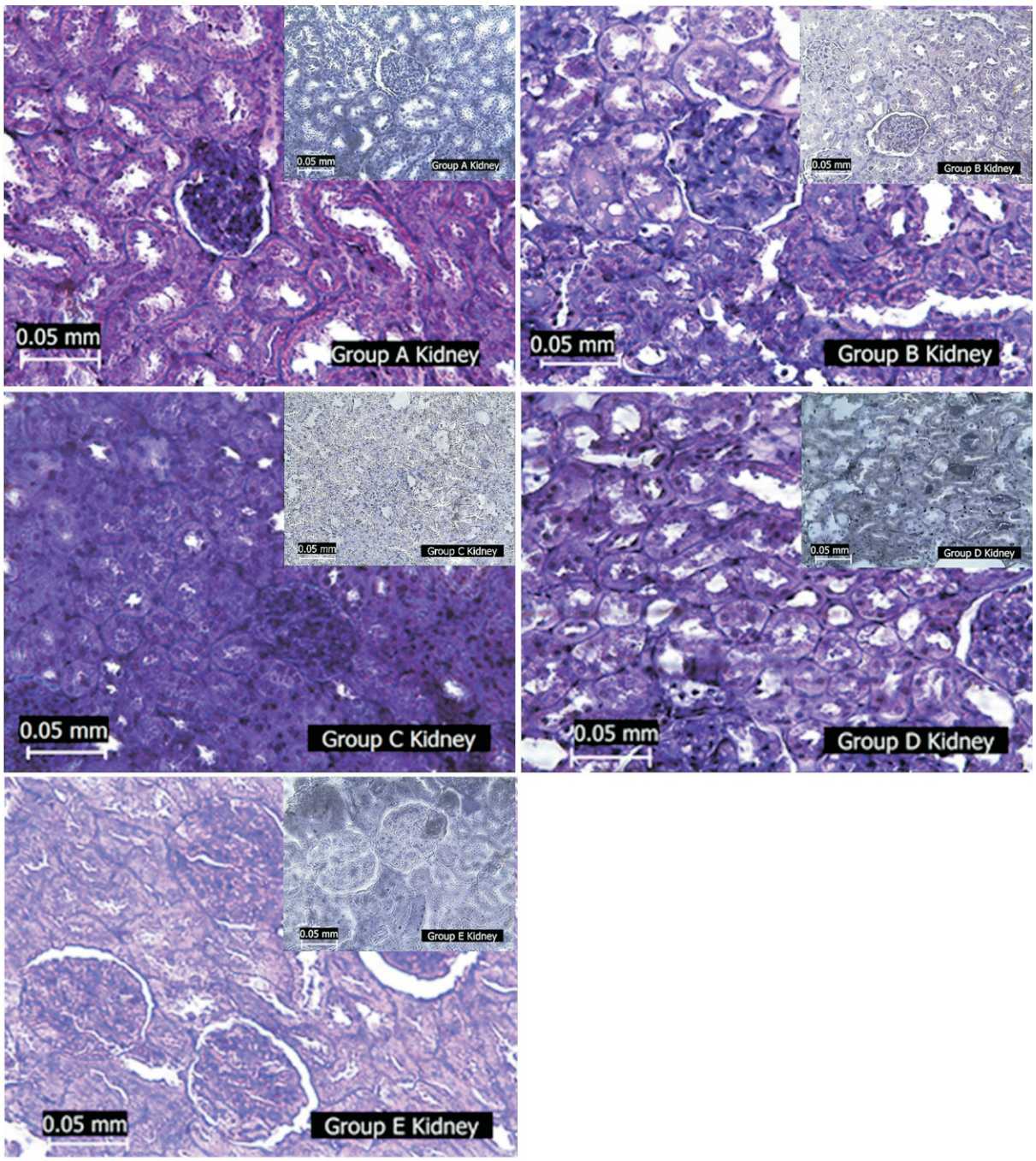


Figure 4: Photomicrographs of section from the cortex of the kidneys of experimental rats (A - normoglycemic rats, B – test group I, C – diabetic negative control, D – test group II and E – diabetic positive control) stained with PAS with diastase control (inset). Glomerular and tubular basement membranes (arrow) and glycogen were well demonstrated in the kidney of normoglycemic and test group II rats. Kidney sections of the diabetic negative and positive control rats showed thickening of basement membrane as well as excessive glycogen deposits. These abnormal features are absent in the kidney of test group I rats.

Histomorphometry: The maximum width of Bowman space in the diabetic negative control group was significantly ($p < 0.05$) lower than that of the normoglycaemic group of rats (Table 6). This diminution persisted in the diabetic positive control group treated with protamin zinc insulin. However, the width of bowman space in the kidney section of the groups treated with HSCE was not significantly different from that of normoglycaemic rats (Table 6). The trans-luminal diameter and cross sectional area of the proximal convoluted tubules were significantly ($p < 0.05$) lower in the diabetic negative and positive control groups than those of the normoglycaemic and the test groups (Table 7). The transluminal diameter of the distal convoluted tubule was significantly higher ($p < 0.05$) in the diabetic negative control group than in the other groups.

Table 6: Kidney Glomerular Morphometry Results

Groups	Maximum Glomerular Diameter (μm)	Maximum Width of the Bowman Space (μm^2)	Number of Glomeruli / unit area of cortex ($N/10^5 \mu m^2$)
A (normal control)	92.13 ± 7.340	8.84 ± 1.275	1.16 ± 0.163
B (Test I)	97.00 ± 4.704	12.12 ± 1.754	1.70 ± 0.177
C (Diabetic -ve control)	110.13 ± 4.552	2.58 ± 0.043*	0.80 ± 0.179
D (Test II)	95.06 ± 2.719	11.83 ± 2.292	1.16 ± 0.213
E (Diabetic +ve control)	112.31 ± 7.234	4.50 ± 0.548 *	0.98 ± 0.126

* $p < 0.05$ compared with the normal control, determined by one way ANOVA followed by DMC *post hoc* test.

Table 7: Kidney Tubular Morphometry Results

Groups	PCT trans - luminal diameter (μm)	DCT trans - luminal diameter (μm)	PCT cross sectional area (μm^2)	DCT cross sectional area (μm^2)	PCT epithelial height (μm)	DCT epithelial height (μm)
A (normal control)	23.99 ± 3.62	32.45 ± 1.54	524.21 ± 35.44	836.35 ± 76.51	17.83 ± 0.14	10.42 ± 0.11
B (Test I)	23.96 ± 1.36	27.77 ± 3.06	461.15 ± 47.00	631.66 ± 89.21	17.73 ± 0.32	11.25 ± 0.40
C (Diabetic -ve control)	10.88 ± 0.63*	40.37 ± 1.50*	95.13 ± 10.27*	1288.76 ± 97.80 *	14.44 ± 1.05	6.59 ± 0.29*
D (Test II)	25.08 ± 0.48	30.16 ± 1.85	495.26 ± 18.45	728.13 ± 91.51	16.92 ± 1.40	8.92 ± 0.90
E (Diabetic +ve control)	16.88 ± 0.73 *	35.20 ± 2.29	226.74 ± 20.32 *	993.88 ± 120.56 *	12.91 ± 2.12*	7.06 ± 0.19*

* $p < 0.05$ compared with the normal control, determined by one way ANOVA followed by DMC *post hoc* test.

DISCUSSION

Several studies have documented that oxidative stress is accelerated in diabetes mellitus owing to an increase in the production of oxygen free radicals, lipid peroxidation and low-density lipoprotein[39]. Free radicals can diffuse intracellularly and result in mitochondrial enzyme damage and DNA breakages, all of which impair cellular function and contribute to the pathophysiology of diabetes[10]. Oxygen free radicals exert their cytotoxic effects on membrane phospholipids, resulting in the formation of MDA. As a product of lipid peroxidation, the levels of MDA reflect the degree of oxidation in the body. As components of the free radical scavenging system, SOD, CAT and GPx exist in all oxygen-metabolizing cells, to prevent damage to cells by free radicals and provide a repair mechanism for oxidized membrane components[40, 41]. In the present study, diabetic nephropathy was significantly improved in rats treated with *H. sabdariffa*. Furthermore, we observed a significant increase in SOD, CAT and GPx activities in *H. sabdariffa*-treated diabetic groups compared with the diabetic positive and negative control groups. The treatment of *H. sabdariffa* also decreased the level of MDA in the diabetic rats. The increase in antioxidants provides effective protection from oxidative damage. The anthocyanins and protocatechuic acid, a phenolic compound present in the extract of *H. sabdariffa* calyx are likely responsible for this strong antioxidant activity.

Histopathological examinations of the kidney sections revealed that STZ diabetic rats as well as insulin-treated diabetic rats manifested with variable extent of kidney damage marked by necrosis of the glomerulus and its infiltration with fats; glomerular enlargement, obliterating the Bowman space, thickening of the glomerular and tubular basement membranes; glycogen deposition in the interstitium and renal interstitial fibrosis, indicated by the presence of collagen in the trichrome-stained kidney sections. Examination of the kidney sections of *H. sabdariffa* extract-treated diabetic rats showed that this extract has nephroprotective activities on the diabetic nephropathy rat models. There was no indication of interstitial fibrosis, glomerular and tubular basement membrane thickening, glomerular necrosis and enlargement in the kidney sections of these extract-treated rats. The result of this study is in agreement with previous reports[42, 43] on the nephroprotective activity of extracts of *H. sabdariffa* on diabetic nephropathy models in rats.

The present study also revealed that renal tubules presented with hydropic change; characterized by pale and swollen change of the proximal convoluted tubules in STZ diabetic rats, as reported previously[44]. This may be a manifestation of osmotic diuresis resulting from high glucose concentration. Administration of *H. sabdariffa* extract significant improved these edematous changes in renal proximal tubules.

Previous studies have shown oxidative stress increasing and anti-oxidative abilities reducing in diabetes[45]. Oxidative stress results in glomerular sclerosis, renal tubular injury, proteinuria and leads to gradual loss of renal function[46]. A detailed pathogenesis of diabetic nephropathy is still not clear. It is considered as primarily a glomerular disease, including thickening of glomerular basement membrane, mesangial expansion and podocyte loss. However, recent evidence demonstrates that chronic hypoxia of the tubule interstitium has a pathogenic role in diabetic nephropathy[47]. In this study, typical diabetic pathological change of Kimmelsteil-Wilson nodules in glomerulus was not found; this might result from too short duration to induce obvious glomerular changes. Indeed, only a minor change had been reported in early diabetic renal tubular injury[48]. Antioxidants generally mitigate any form of oxidative stress or its consequences. They act either by directly scavenging free radicals or increasing antioxidative defenses. A major limitation to the use of antioxidant supplementation is their pro-oxidant activity and potential deleterious effects on ROS production especially when precise modulation of ROS levels are needed to allow normal cell function[49]. The removal of too many ROS and their derived products by antioxidant supplementation may upset the cell signaling pathways and actually increase the risk of chronic disease[50]. The results of this study showed that methanol extract of *Hibiscus sabdariffa*, despite improving antioxidant status in diabetic rats, had no significant effect on normal rats. The extract at the given dose was able to maintain optimal antioxidant status and does not exhibit pro-oxidant effects in normal rats.

The concentration of serum creatinin and urea increased significantly in diabetic rats as earlier reported[51]. However, *H. sabdariffa* extract significantly reduced the serum concentration of creatinin and urea in these rats. This is an indication of improvement in the clearance of these metabolites. In addition, hypoproteinaemia and hypoalbuminaemia were observed in the untreated diabetic rats as previously reported[52, 53]. These conditions, which are closely related to proteinuria were mitigated in the *H. sabdariffa* – treated diabetic rats (Table 4).

CONCLUSION

The results obtained from this study suggest that anthocyanins and protocatechuic acid present in *H. sabdariffa* mitigated experimentally-induced diabetic nephropathy in rat by protecting the kidney from oxidative damage. This study provides an important therapeutic basis for treatment of kidney diseases in insulin deficient diabetes

CONFLICTING INTEREST

The authors declare that they have no competing interests.

FUNDING
The research did not receive any specific grant from funding agencies in public, commercial, or not-for-profit sectors

AUTHORS' CONTRIBUTIONS
ADO; conceived the research idea, designed and executed the experiment and prepared the manuscript. AOS; supervised the research work

ACKNOWLEDGEMENTS
Author is grateful to the authorities of Obafemi Awolowo University Ile Ife for providing laboratory space and equipments for the research, Prof. C.A. Adebajo, department of Pharmacognosy for his useful suggestions and comment and to Mr. Emiola Gbela (retired Chief Technologist, Department of Morbid anatomy and Forensic Medicine, OAU) for his technical assistance in the production of the microscopic slides.

REFERENCES

1. Masharani, U. Diabetes mellitus and hypoglycemia. in: Papadakis, M.A., McPhee, S.J Rabow, M.W. (Eds.) Current medical diagnosis and treatment, fifty-seventh edition, McGraw-Hill, New York, 2018; 1032 - 1073
2. Schrijvers, B.F., de Vriese, A.S., Flyvbjerg, A. From hyperglycemia to diabetic kidney disease: The role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines, Endocrine Reviews 2004; 25 (6), 971 – 1010
3. Chen, H.C., Guh, J.Y., Chang, J.M., Hsieh, M.C., Shin, S.J., Lai, Y.H. Role of lipid control in diabetic nephropathy, Kidney International 2005; 67 Supplement 94, S60 - S62
4. Hovind, P., Tarnow, L., Rossing, K., Rossing, P., Eising, S., Larsen, N., Binder, C., Parving H.H. Decreasing incidence of severe diabetic microangiopathy in type 1 diabetes, Diabetes Care 2003; 26, 1258 – 1264
5. Yokoyama, H., Okudaira, M., Otani, T., Sato, A., Miura, J., Takaike, H., Yamada, H., Muto, K., Uchigata, Y., Ohashi, Y., Iwamoto, Y. Higher incidence of diabetic nephropathy in type 2 than in type 1 diabetes in early-onset diabetes in Japan. Kidney International 2000; 58, 302 – 311
6. Zipp, T., Schelling, J.R. Diabetic nephropathy, in: D.E. Hricik, R.T. Miller, J.R. Sedor (Eds.) Nephrology secrets, Second edition. Hanley & Belfus Inc. Medical Publishers, Philadelphia, 2003; 105 - 111
7. Cooper, M.E., Gilbert, R.E., Pathogenesis, prevention, and treatment of diabetic nephropathy, in R.J. Johnson, J Feehally, (Eds.) Comprehensive clinical nephrology, second edition. Mosby, Edinburgh, 2003; 439-450
8. Williams, M.E., Stanton, R.C. Management of diabetic kidney disease. In Kahn, C.R. Weir, G.C. King, G.L. Jacobson A.M. Moses, A.C. Smith R.J. eds. Joslin's Diabetes Mellitus. Lippincott Williams & Wilkins, Philadelphia. 2005; 925-949
9. Baynes, J.W., Thorpe, S.R. The role of oxidative stress in diabetic complications, *Current Opinion in Endocrinology and Diabetes* 1996; *3,* 277-284
10. Bonnefont-Rousselot, D., Bastard, J.P., Jaudon, M.C., Delattre, J. Consequences of the diabetic status on the oxidant / antioxidant balance, *Diab. Metab.* 2000; *26,* 163-176
11. Turk, H.M., Sevinc, A., Camci, C., Cigli, A., Buyukberber, S., Savli, H., Bayraktar, N. Plasma lipid peroxidation products and antioxidant enzyme activities in patients with type 2 diabetes mellitus, *Acta Diabetol.* 2002; *39* (3), 117-122
12. Cnop, M., Welsh, N., Jonas, J.C., Jörns, A., Lenzen, S., Eizirik, D.L., Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities, *Diabetes* 2005; *54 Suppl.2* S97-S107
13. Simmons, R.A., Developmental origins of diabetes: the role of oxidative stress. *Free Radical Biology and Medicine* 2006; *40*, 917-922
14. Ha, H., Hwang, I.A., Park, J.H., Lee, H.B., Role of reactive oxygen species in the pathogenesis of diabetic nephropathy, Diabetes Res. Clin. Pract. 2008; 82 (Suppl. 1), S42-5
15. Ubani, C.S., Joshua P.E., Oraeki, A.N. Influence of aqueous extract of *Hibiscus sabdariffa* calyces on lipid profile of phenobarbitone induced Wistar albino rats, J. Pharm. Res. 2010; 3, 319-24
16. Liu, C.L., Wang, J.M. Chu, C.Y., Cheng, M.T., Tseng, T.H., *In-vivo* protective effect of protocatechuic acid on tert-butyl hydroperoxide-induced rat hepatotoxicity, Food Chem. Toxicol. 2002; 40 (5), 635–641
17. Ali, B.H., Mousa, H.M., El-Mougy, S., 2003. The effect of a water extract and anthocyanins of *Hibiscus sabdariffa* L. on paracetamol-induced hepatoxicity in rats, Phytother. Res. 17 (1), 56–59
18. Asagba, S.O., Adaikpoh, M.A., Kadiri, H. Obi, F.O. Influence of aqueous extract of *Hibiscus sabdariffa* L. petal on cadmium toxicity in rats, Biol. Trace Elem. Res. 2007; 115 (1) 47 – 57
19. Okoko, T., Oruambo, I.F. The effect of *Hibiscus sabdariffa* calyx extract on cisplatin-induced tissue damage in rats, Biokemistri 2008; 20 (2), 47-52
20. National Research Council (NRC) Institute of Laboratory Animal Research (ILAR), Guide for the Care and Use of Laboratory Animals, eighth edition, National Academy Press, Washington DC, 2011
21. Adeyemi D.O., Adewole O.S. *Hibiscus sabdariffa* renews pancreatic *β*–cells in experimental type I diabetic model rats. Morphologie, 2019; 103: 80 – 93
22. Adeyemi, D.O., Ukwenya, V.O., Obuotor, E.M., Adewole, O.S. Anti-hepatotoxic activities of

Hibiscus sabdariffa L. in animal model of streptozotocin diabetes-induced liver damage, BMC Complementary and Alternative Medicine, 2014, 14 2,77

23. Harborne, A.J., Phytochemical methods: A Guide to Modern techniques of plants Analysis, third edition, Springer, Netherland, 1998, 302 pages
24. Obouayeba, A.P., Djyh, N.B., Diabate, S., Djaman, A.J., N'guessan, J.D., Kone, M., Kouakou, T.H. Phytochemical and antioxidant activity of roselle (*Hibiscus Sabdariffa* L.) petal extracts. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2014; 5(2), 1453 – 1465
25. Adeyemi, D.O., Komolafe, O.A., Adewole, O.S., Obuotor, E.M., Adenowo, T.K., Antihyperglycaemic activities of *Annona muricata* (Linn), Afr. J. Trad. Complement, Altern. Med. 2009; 6 (1), 62 – 69
26. Adeyemi, D.O., Komolafe, O.A., Adewole, O.S., Obuotor, E.M., Abiodun, A.A., Adenowo, T.K., Histomorphological and morphometric studies of the pancreatic islet cells of diabetic rats treated with extracts of *Annona muricata,* Folia morphol. 2010; 69 (2), 92 – 100
27. Drury, R.A.B., Wallington, E.A., Carleton's histological technique, fifth edition., Oxford University Press, London, England, 1980, 520 pages
28. Ohkawa, H., Ohishi, N. Yagi, K., Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 1979; 95(2), 351 - 358
29. Pascual, P., Martinez-Lara, E., Bárcena, J.A., López-Barea, J., Toribio, F., Direct assay of glutathione peroxidase activity using high-performance capillary electrophoresis. Journal of Chromatography. B. Biomedical Sciences and Applications 1992; 581 (1), 49-56
30. Cowell, D.C., Dowman, A.A., Lewis, R.J. Pirzad, R. Watkins, S.D., The rapid potentiometric detection of catalase positive microorganisms, Biosens. Bioelectron. 1994; 9 (2), 131 – 138
31. Kahl, R., Kampkötter, A., W ?tjen, W., Chovolou, Y., Antioxidant enzymes and apoptosis, Drug Metabolism Reviews 2004; 36, 747 - 762
32. Blenn, C., Althaus, F.R., Malanga, M., Poly (ADP-ribose) glycohydrolase silencing protects against H_2O_2-induced cell death. Biochem J. 2006; 396, 419–429
33. Kirkwood, B.R., Sterne, J.A.C. Essential Medical Statistics, second edition, Wiley-Blackwell Science, USA, 2003, 512 pages
34. Beltrán-Debón, R., Alonso-Villaverde, C., Aragonès, G., Rodríguez-Medina, I., Rull, A., Micol, V., Segura-Carretero, A., Fernández-Gutiérrez, A., Camps, J., Joven, J. The aqueous extract of *Hibiscus sabdariffa* calices modulates the production of monocyte chemoattractant protein-1 in humans. Phytomedicine, 2010; 17, 186–191
35. Peng, C.H., Chyau, C.C., Chan, K.C., Chan, T.H., Wang, C.J., Huang, C.N., *Hibiscus sabdariffa* polyphenolic extract inhibits hyperglycemia, hyperlipidemia, and glycation-oxidative stress while improving insulin resistance. Journal of Agricultural and Food Chemistry 2011; 59 (18), 9901–9909
36. Ramirez-Rodrígues, M.M., Balaban, M.O., Marshall, M.R., Rouseff, R.L. Hot and cold water infusion aroma profiles of *Hibiscus sabdariffa*: fresh compared with dried. Journal of Food Science, 2011; 76 (2), 212–217
37. Herranz-López, M., Fernández-Arroyo, S., Pérez-Sanchez, A., Barrajón-Catalán, E., Beltrán-Debón, R., Menéndez, J.A., Alonso-Villaverde, C., Segura-Carretero, A., Joven, J., Micol, V. Synergism of plant-derived polyphenols in adipogenesis: perspectives and implications, Phytomedicine, 19, 253–261
38. Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I., Heinrich, M., 2014. *Hibiscus sabdariffa* L. – A phytochemical and pharmacological review, Food Chemistry, 2012; 165 424–443
39. Reddy, S.V. Tiwari, A.K., Kumar, U.S., Rao, R.J., Rao, J.M. Free radical scavenging, enzyme inhibitory constituents from antidiabetic Ayurvedic medicinal plant *Hydnocarpus wightiana* Blume, Phytother Res. 2005; 19, 277–281
40. Petrulea, M., Muresan, A., Duncea, I., Oxidative stress and antioxidant status in hypo– and hyperthyroidism, in M.A. El- Missiry, (ed) Antioxidant Enzyme, Intech, Croatia, 2012; 197 – 236
41. Lee, W.C., Wang, C.J., Lee, H.J. Antioxidants in decelerating diabetic nephropathy, in A.P. Escher, A Li (eds), Type I Diabetes. Intech, Croatia, 2013, 387 – 399
42. Lee, W.C., Wang, C.J., Chen, Y.H., Hsu, J.D., Cheng, S.Y., Chen, H.C., Lee, H.J. Polyphenol extracts from *Hibiscus sabdariffa* L. attenuate nephropathy in experimental type 1 diabetes, J Agric Food Chem. 2009; 57(6), 2206 - 10
43. Wang, S.C., Lee, S.F., Wang, C.J., Lee, C.H. Lee, W.C., Lee H.J., Aqueous Extract from *Hibiscus sabdariffa* Linnaeus Ameliorate Diabetic Nephropathy via Regulating Oxidative Status and Akt/Bad/14-3-3γ in an Experimental Animal Model, Evidence Based Complementary and Alternative Medicine, 2009; 2011 1 – 9
44. Curran, R.C., Crocker, J. *Curran's Atlas of Histopathology*, fourth edition, Harvey Miller, London, UK, 2000, 288 pages
45. Maxwell, S., Holm, G., Bondjers, G., Wiklund, O. Comparison of antioxidant activity in lipoprotein fractions from insulin-dependent diabetics and

healthy controls, *Atherosclerosis*, 1997; 129, 89–96

46. Shah, S.V., Baliga, R., Rajapurkar, M., Fonseca, V.A. Oxidants in chronic kidney disease, *Journal of the American Society of Nephrology,* 2007; 18 (1), 16 – 28
47. Singh, D.K., Winocour, P., Farrington, K., Mechanisms of disease: the hypoxic tubular hypothesis of diabetic nephropathy, *Nature Clinical Practice Nephrology,* 2008; 4 (4), 216–226
48. Vestra, M.D., Saller, A., Bortoloso, E., Mauer, M., Fioretto, P. Structural involvement in type 1 and type 2 diabetic nephropathy, *Diabetes Metab*. 2000; 26 Supplement 4, 8–14
49. Kuruta, E.B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state, BMC Nutrition Journal 2016; 15, 71
50. Niki, E. Antioxidants: basic principles, emerging concepts, and problems, Biomed J. 2014; 37, 106–11
51. Ravi, K., Ramachandran, B., Subramanian, S., Protective Effect of *Eugenia jambolana* Seed Kernel on Tissue Antioxidants in Streptozotocin-Induced Diabetic Rats, *Biol. Pharm. Bull.* 2004; 27, 1212–1217
52. *Viswanathan, V., Snehalatha, C., Kumutha, R., Jayaraman, M., Ramachandran, A.* Serum albumin levels in different stages of type 2 diabetic nephropathy patients, Indian J Nephrol. *2004;* 14, 89-92
53. Lal, S.S., Sukla, Y., Singh, A., Andriyas, E.A., Lall, A.M. Hyperuricemia, high serum urea and hypoproteinemia are the risk factor for diabetes, Asian Journal of Medical Sciences 2009; 1, 33-34