



PROTECTIVE ROLE OF ERIODICTYOL IN STZ INDUCED DIABETIC NEPHROPATHY IN EXPERIMENTAL RATS

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Abstract

Diabetic nephropathy is an end stage renal disease that has potent involvement of oxidative stress, pro-inflammatory cytokines and many other pathways in its pathogenesis. We hypothesized the Eriodictyol, a bio flavanone could protect from ensuing renal impairment in diabetes by altering the oxidative stress and inflammatory pathways in renal cells. The aim of study is to investigate the nephro-protective effect of Eriodictyol in STZ induced diabetic experimental rats. Eriodictyol treatment for four weeks in two doses (1mg/kg and 10mg /kg) post induction of diabetic nephropathy, highly significantly ameliorates the renal functions impairment, inflammatory cascades and impaired oxidative stress. Eriodictyol also restores the kidney weight, kidney index, feed/water intake and body weight. Eriodictyol significantly attenuates the lipid profile by restoring the LDL, VLDL, HDL, TC and TGs level in serum. Eriodictyol 10 mg /kg treatment also provides a remarkable effect on Advanced Glycation End Products generation in renal tissue. Moreover, Eriodictyol also provides diminution of the pro inflammatory cytokines like IL-6, TNF- α , TGF- β 1 synthesis in kidney homogenate. These results illustrate that Eriodictyol exhibits the kidney protective effect in experimental rats by playing the antihyperglycemic role by increasing the insulin level in blood which was accompanied by diminution of oxidative and inflammatory cascades.

Keywords: Diabetic Nephropathy, Eriodictyol, STZ etc.

Introduction

The prevalence of diabetes and its complications among all metabolic disorders is gradually increased during 21th century throughout the world (1). Over a long period of practice, there is not any remarkable treatment for diabetic nephropathy and other complications (2). According to International Diabetic Federation (IDF), diabetes plays a considerable burden on public health and their economic status. According to IDF diabetes atlas 10th edition 2021, 537 million adults (20-79) years are living with diabetes. Diabetes is responsible for 6.7 million deaths in 2021 that is 1 every 5 seconds and this figure is anticipated to increase 783 million till 2045. 541 million adults have impaired glucose tolerance (IGT), which place them at high risk of type 2 diabetes. The overall prevalence of diabetes and its complication is above 10% of all diseases (3). In 2019, India has reported to have diabetic patients about 77.0 million. It has also been estimated that India will have 134.2 million diabetic patients with complication by 2045 (4, 5). Hyperglycemia plays a crucial role in development of diabetic nephropathy. Prolonged and uncontrolled hyperglycemic level in blood leads to chronic kidney diseases and then

mediates diabetic nephropathy. Hyperlipidemia is an initial step for nephropathy. Elevated level of Cholesterol, triglyceride and fatty acid starts to deposit in non-adipose tissue like muscles, liver kidney (6). This hyperlipidemia gradually decreases the glomerular filtration rate (GFR) accompanied albumin urea by activating various biochemical and intracellular pathways. The activation of these pathways leads to glomerulosclerosis, endothelial dysfunction, oxidative stress, mesangial expansion, podocyte loss and albuminuria, and tubulointerstitial damage (7).

Albumin is also a potent biomarker for detection of nephropathy. The presence of albumin in urine is also associated with decreasing glomerular filtration rate. Level of microalbumin urea and macroalbuminuria with time shows the degree of progressions of nephropathy (8). Advanced glycation end products also play an important role in pathogenesis of diabetic nephropathy. Excessive reduced sugar combines to lipid, protein, fats non enzymatically through glycation reaction. AGEs have capability to promote inflammation, oxidative stress and apoptosis (9,10). These AGEs products bind to receptors of advanced glycation end products (RAGEs) and initiate the inflammatory reactions. AGEs activate inflammatory cytokines (IL-6, TNF- α , TGF- β 1) and increase their production, which leads to inflammatory reactions in body. These AGEs also give rise to reactive oxygen species (ROS) which leads to hypertrophy and apoptosis of glomerulus and podocytes respectively (11).

Eriodictyol ((2S)-2-(3, 4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydrochromen-4-one) is a bioflavonoid of flavanone subclass abundantly found in citrus fruits, vegetables, and medicinal plants (12). Its molecular weight is 288.25 g/mol and molecular formula is C₁₅H₁₂O₆. Commercially, it is extracted from Yerba Santa (*Eriodictyon californicum*) plant (13). Flavanones are a subgroup of flavonoids characterized by two aromatic rings (A and B) linked by a dihydropyrone ring C. Eriodictyol is a polyphenolic tetrahydroxy flavanone compound. The structural variations of citrus flavonoids such as hydroxyl forms of A and B rings and the presence of 2, 3-unsaturation infused with a 4-oxo group in the C-ring have an impact on their beneficial biological properties (14).

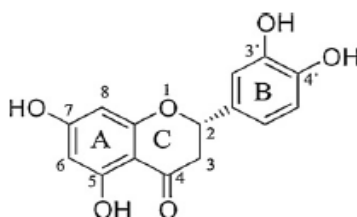


Figure 1: Eriodictyol Structure

Eriodictyol shows its antioxidant activity because the presence of high number of hydroxyl groups (15). It has been reported that flavonoids which inhibit tyrosine kinase and protein kinase C, have 3', 4'-OH groups on the B ring, and 5, 7-OH groups on the A ring (16, 17). In C ring, presence of 4-oxo group also contributes in suppression of COX-2 transcriptional activity by which it shows the anti-inflammatory activity (18). In B ring, the presence of a double bond between C-2 and C-3 may be very important for Phosphodiesterase 2 (PDE2) inhibition which is responsible for insulin secretion (19, 20). Due to its potent anti-inflammatory (21) and antioxidant (22) activity, it is also reported for anti-diabetic activity by inhibiting AGE products *in vitro* (23). It shows protective role of diabetic retinopathy by restoring TNF- α , ICAM-1, VEGF, and eNOS level in ganglial cells (24). It also reveals the insulinotropic impact on glucose via cAMP/PKA pathway *in vitro* (25). It reports the protective effect on mesangial cell in high blood glucose level by inhibiting Akt/NF- κ B pathway through TNF- α , IL-1 β , and IL-6 cytokines *in vitro* studies (26). Eriodictyol also have potent anticancer (27), hepatoprotective activity (28), neuroprotective (29), cardioprotective activity (30). This study explored the *in vivo* protective effect of Eriodictyol against diabetic nephropathy.

Material and Methods

Drugs and Chemicals: Eriodictyol (purity >98%, CAS-552-58-9) was procured from Biosynth Carbosynth Limited, UK. Streptozotocin was procured from Sigma Aldrich (USA), Metformin from Abbott India Limited. Creatinine (Cr), triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL-C) and blood urea nitrogen (BUN) colorimetric detection kit procured from Elabscience. Advanced Glycation End Products (AGEs) ELISA kit from Cusabio. HBA1c procured from Creative Diagnostic Kit. Tumor necrosis factor - α (TNF- α), interleukin-6 (IL-6), rat albumin ELISA kit (Bethyl Laboratories), rat TGF- β 1 (transforming growth factor beta 1) ELISA kit (Elabscience) was procured. All other reagents used in study, were of analytical grade and purchased from Himedia Labs, Mumbai (India).

Drug Preparations: Eriodictyol dissolved in 5% Tween 80, 20% PEG and 75% Saline (24). STZ was freshly prepared in 0.1 M cold Citrate buffer pH 4.5 (31). Metformin solution was prepared with distilled water.

Experimental Animals: Adult male Wistar rats (6 weeks of age) weighing 200-250 gm were purchased from CPCSEA approved registered breeder Central Animal Facility, AIIMS, Delhi. The protocol for antidiabetic activity was approved by Institution Animal Ethics Committee (IAEC) of Swift College of Pharmacy, Ghaggar Sarai, Rajpura, Patiala, Punjab (Reg No.

724/PO/a/02/CPCSEA) with protocol No SSP/IAEC/2022/06 on dated 9/06/2022. The animals were housed under standard laboratory conditions with temperature ($23 \pm 1^\circ\text{C}$), relative humidity ($55 \pm 10\%$), 12/12 h light /dark cycles and fed with standard pellet diet from Ashirwad Industry and purified water *ad libitum*. All rats were free to drink clean water and normal pallet diet during the experimental period.

Induction of Diabetic Nephropathy: After acclimatization of two weeks, adult, albino wistar rats kept fasted overnight. Next morning, freshly prepared STZ 55mg/kg was injected intraperitoneally to animals (31, 32). After 72 hours, blood glucose level was checked with glucometer by collecting the blood sample from tail vein and diabetes was confirmed (33). The rats with more than 250mg/dl were considered diabetic. The sustained level of glucose for 8 weeks induced diabetic nephropathy (31, 32).

Experimental Design

Forty rats were divided in to two groups randomly i.e., vehicle treated group (8 rats) and streptozotocin (STZ) treated group (remaining animals).

Non diabetic animals	Group 1 (Control Group) - Rats received single injection of cold citrate buffer
Diabetic animals	Group 2 (Diabetic Nephropathy Group) - Streptozotocin (STZ) 55 mg/kg i.p. at the 1 st day
	Group 3 (ED1 Group) - STZ injected rats received Eriodictyol (1 mg/kg, orally) for 28 days after 8 weeks
	Group 4 (ED10 Group) - STZ injected rats received Eriodictyol (10 mg/kg, orally) for 28 days after 8 weeks
	Group 5 (Metformin group) - STZ injected rats received metformin (200 mg/kg, orally) for 28 days after 8 weeks

Metformin dose was used 200mg/kg (34). Eriodictyol was given in 2 doses *i.e.* 1 mg and 10 mg/kg orally to different groups. STZ mediates various pathways in the renal tissue and causes diabetic nephropathy (35). Feed intake, water intake and body weight were noted every week during experimental period.

Scarification and Specimen Collection: After twelve weeks of diabetic nephropathy study, the rats were sacrificed by an overdose of thiopental sodium (40 mg/kg). Blood samples were collected from orbital sinus and centrifuged to separate serum which was used immediately for assessment of biochemical parameters, inflammatory biomarkers and lipid level or store the serum for later analysis at 80°C. The animals' body weights were recorded. Both kidneys were harvested, rinsed in cold saline and weighed them for calculation of kidney index= (kidney weight/body weight) x 1000 to check the rate of hypertrophy in kidney. Kidney specimens

were frozen at -80°C for preparation of kidney homogenate, for oxidative stress level estimation.

Estimation of Biochemical Parameters: Blood sample was kept for 2 hours at room temperature or at $2-8^{\circ}\text{C}$ for overnight. Then the sample was centrifuged for 20 min. at 1000 rpm at 4°C . Supernatant was collected and different procedures for serum albumin, serum creatinine, HbA1C and insulin before and after treatment were carried out. These all were calculated, according as per given manufacturer instructions on kits. Blood urea nitrogen (BUN) and glycated hemoglobin (HbA1C) was assayed by ELISA test kit and optical density (OD) of sample was noted at 550 nm and 450 nm respectively. Serum creatinine (SCr) content in sample was checked by Sarcosine Oxidase Method. OD of sample for serum creatinine was measured by biochemical analyzer at 515 nm.

Lipid Profile Estimation: The centrifuged blood (for 20 min. at 1000 rpm at 4°C) was used to carry out assay by collecting the supernatant fluid. Lipid profile was estimated by colorimetric method with help of biochemical analyser and spectrophotometer. Assessment of Total-cholesterol (TC), triglycerides (TGs), LDL cholesterol (LDL-C), VLDL cholesterol (VLDL-C), and HDL cholesterol (HDL-C) was done as per instructions given by manufacturer on kits.

Advanced Glycation End Products Estimation: AGEs estimation was done with ELISA test kit. 100 mg of harvested kidney was rinsed with 1X phosphate buffer saline (PBS), homogenized in 1 ml of 1X PBS and stored overnight at -20°C . After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 rpm, $2-8^{\circ}\text{C}$. The supernatant was removed and assayed immediately. Alternatively, aliquot and store the samples at -20°C or -80°C , centrifuge the sample again after thawing before the assay. Incubate the sample at 37°C . Protect it from light. Check the reading at 450 nm within 5 minutes.

Estimation of Antioxidants and Lipid Peroxidation: The excised kidney, rinsed in ice cold normal saline followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10% w/v of homogenate was prepared in 0.15 M Tris-HCl buffer and processed for the estimation of lipid peroxidation (TBARS) by the method of Ohkawa H *et al* (36). A part of homogenate after precipitating proteins with Trichloric acetic acid (TCA) was used for estimation of glutathione (GSH) by the method of Ellman GL *et al* (37). The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4°C . The supernatant thus obtained was used for the estimation of Catalase activities was measured by the method of Claiborne *et al* (38). Nitrites level assessed

by using copper Cadmium alloy to reduce nitrate to nitrite in an hour followed by Griess reagent by colour development in acidic medium (39). 100 μ L supernatant from the kidney homogenate is thoroughly mixed 100 μ L Griess reagent. Check the absorbance at 540 nm and compared from the standard curve of sodium nitrite (0-100 μ L). Super Oxide Dismutase assessment (SOD) was based on its ability to inhibit the reduction of nitro blue tetrazolium (NBT) by using sodium carbonate buffer having 0.1 mM xanthine, 0.1 mM EDTA, xanthine oxidase, 0.025 Mm NBT (40).

Estimation of Inflammatory Cytokines: Samples was allowed to clot for 2 hours at room temperature or overnight at 4°C before centrifugation for 20 min at 1000 rpm at 2~8°C. The supernatant was collected to carry out the assay. Blood collection tubes should be disposable and be non-endotoxin. Remove serum layer and assayed immediately or store serum samples at temperature < -20°C. Repeated freeze/thaw cycles were avoided. IL-6 was estimated by using the Sandwich-ELISA principle. The reaction was terminated by showing the yellow colour after adding stop solution. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm \pm 2 nm. The OD value is proportional to the concentration of Rat IL-6. IL-6 concentration was calculated by comparing the OD of the samples to the standard curve. TGF- β 1 was estimate by Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit. Inactive TGF- β 1 in sample was first turned to activate. The reaction was terminated by showing yellow colour. The O.D. absorbance read with a microplate reader at 450nm, within 30 minutes of stopping the reaction. TNF- α was estimated by using ELISA kit, by noted the absorbance at 450 nm within 30 minutes of stopping reaction.

Statistical Analysis: Statistical analysis was done by using Graph Pad 6. Means of normally dispersed variables were examined and followed by one or repeated measures two -way analysis of variance (ANOVA). If ANOVA conclusions were significant ($p < 0.05$) in F-statistics, multiple comparison tests viz. then Tukey's HSD (Honest Significant Difference) test or Bonferroni test were used. Statistical significance was estimated at $p < 0.05$ and results were stated as mean standard deviation.

Results

Effects of Eriodyctiol on Physical Parameters: Body weight of rats usually increased with age because of increased body mass. STZ treated animals (DNP group) showed a significant decrease in body weight when compared with control group animals. Treatment with Met (Metformin) and ED (Eriodyctiol) restored the body weight in comparison to DNP (Diabetic Nephropathy) group animals (Fig.2C) . ED10 (Eriodyctiol 10 mg/kg) showed a better weight

restoration in comparison to ED1 (Eriodictyol1 mg/kg). Feed intake of rats of control group was observed constant during the study period. STZ treated animals showed gradually increase in food intake when treatment was given to diabetic rats, a decline was observed in food intake (Fig. 2B). ED10 and Met highly significantly decreased the food intake capacity of rats in comparison to DNP group animals. Diabetes also affects the water intake in rats. A gradually increase in water intake was observed in STZ treated rats (Fig. 2A). Met treated diabetic rats showed a significant decrease in thirst in comparison to DNP group rats. ED10 also showed a significant decrease in water intake when compared with DNP group and ED1 treated group rats.

Kidney index is the ratio of kidney weight by body weight. Kidney weight was also increased with kidney index when rats were injected with STZ. So, there was a significant increase in kidney index was found in STZ treated rats. Metformin and Eriodictyol treatment restored the kidney weight (Fig.2 D) as well as kidney index (Fig.2 E). ED10 showed a highly significant decrease in kidney index when compared with DNP group and ED1 treated group.

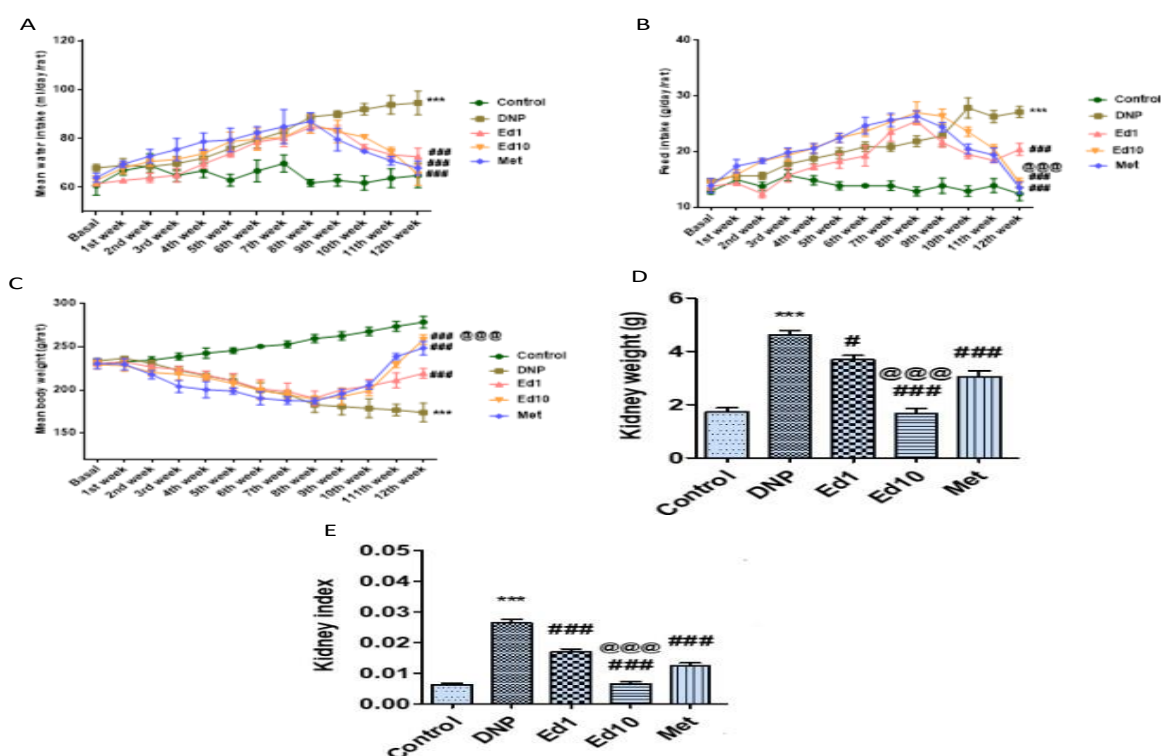


Fig.2. Effect of ED on (A) mean water intake, (B) mean feed intake, (C) mean body weight, (D) Kidney weight, (E) Kidney index. Data of water/feed intake and body weight were analyzed using a repeated measure two-way ANOVA and Bonferroni post hoc test. One way ANOVA followed by Tukey's test HSD was used to analyze kidney weight and kidney index.

Data explored as mean \pm S.E.M, (n=8) symbols represent statistical significance.*** Vs Control,^{###} Vs DNP, ^{@@@} Vs ED1, $p^{***} = <0.001$, $p^{###} = <0.001$, $p^{##} = <0.01$, $p^{\#} = <0.05$, $p^{@@@} = <0.001$. DNP- Diabetic Nephropathy, ED1- Eriodictyol 1 mg/kg, ED10- Eriodictyol 10 mg/kg, Met- 200mg/kg.

Effect of Eriodictyol on Serum Parameters: Serum creatinine, HbA1C and BUN measured during the study and showed a highly significant increase in their level in diabetic nephropathy group when compared with control group (Table-1). Standard drug metformin showed significant decrease in the level of Serum creatinine, HbA1C and BUN in comparison with diabetic nephropathy group. Eriodictyol treatment in both doses (ED1mg/kg and ED10 mg/kg) revealed a significant increase in their level when compared with diabetic nephropathy group. ED 10 group also showed a highly significant increase in the level as compared to ED1 group. Whereas the serum albumin level and insulin level were observed highly decreased when compare of diabetic nephropathy with normal control group (Table-1).ED 10 treated animals showed a significant increase in insulin and albumin level as compared to ED 1 and Metformin treated animals.

Table 1 Effect of Eriodictyol on Biochemical Parameters

GROUPS	Serum Albumin (ng/mL)	Serum Creatinine (μ mol/L)	BUN (mmol/L)	HbA1C (ng/mL)	Insulin (BT) (μ IU/mL)	Insulin (AT) (μ IU/mL)
CONTROL GROUP	294.4 \pm 20.7	96.6 \pm 8.2	5.1 \pm 2.2	13.6 \pm 3.3	18.3 \pm 1.4	18.2 \pm 1.1
DNP GROUP	88.2 \pm 32.9 ^{***}	353 \pm 47.9 ^{***}	20.1 \pm 2.7 ^{**} *	68.5 \pm 9.5 [*] **	9.1 \pm 1.8 ^{***}	8.6 \pm 2 ^{***}
ED 1	141.2 \pm 25.1 ^{##}	289 \pm 36.5 [#]	15.1 \pm 4.4 [#]	51.3 \pm 4.6 [#] ##	10.5 \pm 1.6 ^{***}	12.4 \pm 2.5 ^{##}
ED 10	282.5 \pm 16.7 ^{###} @@@	160.1 \pm 58.5 ^{##} #@#@	9.1 \pm 2.8 ^{###} @@	25.5 \pm 7.2 [#] ##@@@	9.3 \pm 1.2 ^{***}	17.1 \pm 1.3 ^{###} @@
MET	260.1 \pm 27.7 ^{###}	157.8 \pm 20.8 ^{##} #	10.3 \pm 3.4 ^{##} #	33.2 \pm 6.2 [#] ##	9.1 \pm 1.2 ^{***}	17.2 \pm 1.1 ^{###}

Value Mean \pm S.E.M, (n=8) symbols represent statistical significance.*** Vs Control,^{###} Vs DNP,^{@@@} Vs ED1, $p^{***} = <0.001$, $p^{###} = <0.001$, $p^{##} = <0.01$, $p^{\#} = <0.05$, $p^{@@@} = <0.001$. One way ANOVA followed by Tukey's HSD Multiple Comparison test. DNP= Diabetic Nephropathy, ED1= Eriodictyol 1 mg/kg, ED10=Eriodictyol 10 mg/kg, BUN= Blood Urea Nitrogen, BT= Before Treatment, AT= After Treatment, HbA1C= Glycated Hemoglobin, AGEs= Advanced Glycation End Products.

Effect of Eriodictyol on Lipid Profile: A highly significant increase in the TC, TGs, VLDL and LDL-C levels in DNP group animals compared with control group animals whereas HDL-C level was observed decreased (Table-2). ED 10 and Metformin treated groups showed a highly significant decrease in their value except HDL-C level. HDL-C level was restored in ED 10 and Metformin treated group. ED 10 showed a highly significant hike in HDL-C level when compared with ED 10 and Metformin.

Table 2 Effect of Eriodictyol on Lipid Profile

GROUPS	TC (mg/dL)	TGs (mg/dL)	VLDL (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)
CONTROL GROUP	5.1±2.4	2.1±1.2	291.3±60.7	2.5±0.6	1.2±0.4
DNP GROUP	18.1±2.2***	9.2±1***	912.1±162.8***	0.9±0.1***	9.1±0.7***
ED 1	14.2±2.7 [#]	7.3±1 [#]	746.1±140.6 [#]	1.3±0.4 [#]	7.3±1 [#]
ED 10	5.2±1.6 ^{###@@@}	3.1±1.1 ^{###@@@}	334.6±51.7 ^{###@@@}	2.3±0.6 ^{###@@@}	2.1±0.6 ^{###@@@}
MET	7.3±1.6 ^{###}	6.1±0.6 ^{###}	506.3±83.4 ^{###}	1.5±0.4 ^{###}	5.3±1 ^{###}

Value Mean ± S.E.M, (n=8) symbols represent statistical significance. *** Vs Control, ### Vs DNP, @@@ Vs ED1, $p^{***} = <0.001$, $p^{###} = <0.001$, $p^{##} = <0.01$, $p^{#} = <0.05$, $p^{@@@} = <0.001$. One way ANOVA followed by Tukey's HSD Multiple Comparison test. DNP- Diabetic Nephropathy, ED1- Eriodictyol 1 mg/kg, ED10- Eriodictyol 10 mg/kg, VLDL= Very Low-Density Lipoprotein. HDL-C= High Density Lipoprotein- Cholesterol, LDL-C= Low Density Lipoprotein-Cholesterol, TC=Total cholesterol, TGs= Triglycerides.

Effect of Eriodictyol on Antioxidants and TBARS

Catalase, GSH and SOD are the enzymes that possess the antioxidant activity against various reactive oxygen species. In DNP group, there was a remarkable increase in these enzymes level (Table-3). ED1 group animals showed a less significant increase in their level on comparison with DNP group animals. Met group animals also showed a highly significant increase in the level on comparison with DNP group. ED10 revealed a potent antioxidant activity and significantly improvement in their level when compared with DNP group and ED1 group animals. Lipid peroxidation and nitrites level were comparatively increased in DNP group animals as compared to control group animals (Table-3). ED1 showed a less significant whereas ED10 and Metformin group animals showed highly significant restoration of LPO and nitrites level in animals.

Table 3 Effect of Eriodictyol on Antioxidants and TBARS

GROUPS	CATALASE (units /mg tissue)	TBARS/LPO (nmol/mg)	SOD (U/mg)	GSH (μmol/mg)	NITRITES (μmol/mg)
CONTROL GROUP	66.2±11.6	14.2±5.4	37.2±5.9	102.1±9.7	16.1±5.2
DNP GROUP	22.4±3.2***	60.4±9.9***	17.1±4.1***	30.2±9.6***	75.2±6.7***
ED 1	33.1±9.1 [#]	48.1±7.6 [#]	25.1±3.3 [#]	45.2±9.2 [#]	65.3±9.1 [#]
ED 10	65.2±4.6 ^{###@@}	21.3±6.1 ^{###@@}	37.2±7.5 ^{###@}	95.3±8.2 ^{###@}	27.2±4.1 ^{###@@@}
MET	56.4±3.9 ^{###}	29.1±5.3 ^{###}	31.3±6.7 ^{###}	85.2±11.3 ^{###}	30.1±5.7 ^{###}

Value Mean ± S.E.M, (n=8) symbols represent statistical significance.*** Vs Control,### Vs DNP, @@@ Vs ED1, p***= <0.001, p###=<0.001, p##=<0.01, p#=<0.05, p@@@=<0.001. One way ANOVA followed by Tukey's HSD Multiple Comparison test. DNP= Diabetic Nephropathy, ED1= Eriodictyol 1 mg/kg, ED10= Eriodictyol 10 mg/kg, SOD- Superoxide Dismutase, TBARS/LPO= Thiobarbituric acid reactive substances/Lipid Peroxidation, GSH= Glutathione.

Effect of Eriodictyol on Inflammatory Cytokines

IL-6, TGF-β1, TNF-α are some inflammatory cytokines which were measured in all group's animals. Diabetic nephropathy (DNP) group showed a significant increase in their value on STZ administration when compared with control group animals. ED1 and treated animals showed a less significant and high significant improvement (Table-4) respectively in their levels on comparison to DNP group animals. ED10 treated animal group showed a high significant improvement in inflammatory cytokines level when comparison was done with DNP group and ED1 group.

Effect of Eriodictyol on AGEs

Advanced Glycation End products are the results of reduced sugar glycation reaction in body in hyperglycemic state. The level of AGEs was high significantly increased in diabetic rats STZ injected rats as compared to control group (Table-4). Metformin, showed a highly significant decrease in the level of AGEs when compared with DNP group rats. ED10 treated animals was observed with a highly significant decrease in AGEs level when comparison was done with ED1 and DNP group animals.

Table 4 Effect of Eriodictyol on Inflammatory Cytokines and AGEs.

GROUPS	IL-6 (pg/ml)	TGF-β1 (pg/ml)	TNF-α (pg/ml)	AGE (μg/ml)
CONTROL GROUP	58.1±14	61.03±27.6	67.2±10.6	28.14±6.2
DNP GROUP	275.2±35.7 ^{***}	280.7±25.9 ^{***}	188.6±35.6 ^{***}	147.8±30.2 ^{***}
ED 1	226.9±33.3 [#]	221±23.3 ^{##}	147.1±21.4 ^{##}	110.5±28.2 ^{##}
ED 10	85.2±16.3 ^{###@@@}	90.2±29.6 ^{###@@@}	86.4±19 ^{###@@@}	53.4±16.3 ^{###@@}
MET	134.8±27 ^{###}	121.3±20.7 ^{###}	97.7±11.1 ^{###}	80.7±15.4 ^{###}

Value Mean ± S.E.M, (n=8) symbols represent statistical significance. ^{***} Vs Control, ^{###} Vs DNP, ^{@@@} Vs ED1, $p^{***} < 0.001$, $p^{###} < 0.001$, $p^{##} < 0.01$, $p^{\#} < 0.05$, $p^{@@@} < 0.001$. One way ANOVA followed by Tukey's HSD Multiple Comparison test. DNP= Diabetic Nephropathy, ED1=Eriodictyol 1 mg/kg, ED10=Eriodictyol 10 mg/kg, IL-6= Interlukin-6, TNF-α= Tumor Necrosis Factor- alpha, TGF-β1=Transforming Growth Factor-beta.

Discussion

Uncontrolled and long-term exposure to high glucose changes the systematic functioning of kidney and leads to podocyte damage, endothelial-mesangial transition, glomerosclerosis, GFR, kidney sclerosis and fibrosis (7). STZ induced diabetic nephropathy is the well-established and known model of diabetes mellitus in experimental rats. STZ selectively destroy the pancreatic β cells by entering through GLUT -2 receptors. It mediates β cell necrosis by stimulating the DNA damage via various pathways like PARP-1, by depletion of NAD⁺ and ATP intracellularly (41, 42). After administration, STZ itself breakdown into glucose and methylnitrosourea which is responsible for molecular changes in cell by fragmenting DNA, alter biological reactions in β cells of pancreas (41).

In current study, STZ model is used to induce diabetes from one time dose of STZ (55 mg/kg *i.p.*) and sustained hyperglycaemic condition for 8 weeks, responsible for induction of diabetic nephropathy. Along with hyperglycaemia, STZ also causes catabolic effect on protein and fat and responsible for increased body weight, by causing polyphagia, polyuria, polydipsia (43,44). Here in this study, Eriodictyol is used to treat diabetic nephropathy. After 4 weeks treatment with Eriodictyol, the glucose utilization was improved in blood and body weight, water/feed intake gradually decreased. Eriodictyol restore the renal electrolyte balance by improving Na-K-ATPase pump and provide significant improvement in polydipsia and polyphagia. Kidney index (kidney weight/body weight) is increased in DN, because of

hypertrophy and hyperplasia of renal cells (45). Eriodictyol also showed a regression of kidney index in experimental animals.

Albuminuria is the potent biomarker of diabetic nephropathy. Macro and microalbumin are present in urine hypoalbuminemia (46). Production of non-nitrogenous base compound from the protein catabolism and nitrogen imbalance in body, leads to formation of BUN and creatinine. The increase in level of BUN, creatinine and albumin in blood indicates the diabetic nephropathy by altering the glomerular filtration rate. In current study, diabetic nephropathy is assured by measuring the level of all these parameters. Eriodictyol shows a significant role in restoring the level of BUN, creatinine and by increasing albumin level in blood against diabetic nephropathy. Diabetic nephropathy is characterized by not only dysfunction of glucose metabolism but also dyslipidaemia. Dyslipidaemia occurs due to increase breakdown of lipid and free fatty acids. Insulin is responsible for lipogenesis and inhibits lipolysis, removal of LDL. In DN, insulin secretion is decreased, resulting decreased lipogenesis and promote lipolysis and increase the deposition of LDL, VLDL. Lipid metabolism is also altered by impairment of lipoprotein lipase enzyme in endothelial cell and responsible to increase level of TGs, TC, VLDL-C, LDL-C and decrease HDL-C level (50). Eriodictyol improves lipid metabolism, resulting in decreased TGs, TC, VLDL-C, and LDL-C and increased HDL-C level.

Oxidative stress plays the key role in pathogenesis of diabetes mellitus by the excess production of reactive oxygen species in hyperglycemic state. Nicotinamide adenosine dinucleotide phosphate (NAPDH) oxidase and mitochondrial dysfunctioning are two major sources of ROS in diabetic kidney. The excessive production of ROS causes DNA damage, cell damage and other pathological changes in tissue. The sustained ROS (H_2O_2 , HO^*) resulting damage of renal tissue and leads to lipid peroxidation. In present study, decrease of SOD, GSH, catalase enzymes by Eriodictyol are observed which are responsible for removal of redox ions in DN. There are two pathways which are responsible for reactive oxygen species (ROS) formation from glucose *i.e.* enzymatic and non-enzymatic. Glucose is oxidised to reactive superoxide ions. SOD and Catalase are the defence system of cells against reactive ion species and scavenge by converting them into non-reactive species. GSH provides enzymatic protection against ROS by absorbing HO^* from H_2O_2 and neutralises by convert it into water. Hyperglycaemic condition impaired these reactions by increasing the amount of ROS. TBARS (Thiobarbituric Acid Reactive Species) are the potent compound to measure the level of lipid peroxidation in cell or tissue. The reactive oxygen species which are generated from lipid

peroxidation, are main cause of cell damage. TBARS level in blood is increased in hyperglycaemic condition in diabetes mellitus . Due to the inappropriate antioxidant defence system, in DN condition, SOD, GSH and catalase activity start to decline and lipid peroxidation starts to increase. Eriodictyol showed a proper scavenging activity against reactive oxygen species and provide a significant protection from oxidative stress. Eriodictyol improve the SOD, GSH and catalase activity reduced the TBARS level by reducing lipid peroxidation on 4 weeks treatment.

AGEs are the products of glycation of reduced sugar in hyperglycemic condition. Excessive formation of AGEs mediates the progression of diabetes to diabetic nephropathy. Detection of AGEs in blood stream is the major sign of renal impairment . Increased AGEs level is also found in other types of diabetic complications, but it is more prominent in diabetic nephropathy. Kidneys are more sensitive in formation of AGEs. Irreversible AGEs formation leads to blood vessels damage and play an important role in pathogenesis of nephropathy. On STZ administration, a hike of AGEs level is found in rat blood. After 4 weeks treatment with Eriodictyol, it showed a remarkable decline in level of AGEs. It improves the hyperglycemic condition and reduces the availability of sugar for glycation process and avoids the excess formation of AGEs.

Oxidative stress is also connected to inflammatory response of cell in kidney. Anti-inflammatory and antioxidant activity of cell are very important to prevent the cell and tissue from the diseases. These activities of cell helps in restore the kidney structure and functions. In this study, pro inflammatory cytokines are measured in blood. These proinflammatory cytokines are the mediators to active the cell inflammatory response. If the level of proinflammatory cells found high then cells are prone to activate inflammatory reaction.

IL-6, TNF- α , TGF- β 1 is some proinflammatory cytokines, which are considered to activate the inflammatory activity in kidney cells. Glomerulus of kidney are very sensitive to wards TNF- α , it plays a cytotoxic role for glomerulus cells. It decreases the filtration rate of mesangial cells by impairing glomerular filtration and damages the epithelial cells in kidney tubules and glomerulus. IL-6 is responsible for glomerulosclerosis and acute and chronic nephritis, in diabetic nephropathy. Over expression of TNF- α can lead to over production of IL-6. TGF- β 1 is the cause of synthesis and accumulation of extracellular matrix protein (fibronectin, collagen) in glomerular cells, resulting glomerular basement membrane thickening. Sustained hyperglycemic condition leads to over expression of these inflammatory cytokines and leads to hypertrophy and fibrogenesis of kidney resulting impaired filtration rate. STZ induces the

inflammatory response of cell by depressing its anti-inflammatory activity and leads to diabetic nephropathy. Eriodyctiol showed a marked improvement in anti-inflammatory activity by decreasing the blood level of IL-6, TNF- α and TGF- β 1.

Conclusion

Eriodyctiol reduced the pathological characters of diabetic nephropathy in dose dependent manner. Its high dose (10mg/kg) showed a remarkable diabetes nephroprotective effect which was induced by single dose of STZ. Eriodyctiol ameliorates the nephroprotective pathways like antioxidant, anti-inflammatory in DN rats. It showed highly significant results when comparison was done with pre-existing drug Metformin. There is further scope of Eriodyctiol, to provide a promising alternative treatment to diabetes nephropathy.

References

1. Shao, Y., & Shi, X. (2023). Bibliometric Analysis and Visualization of Research Progress in the Diabetic Nephropathy Field from 2001 to 2021. *Oxidative medicine and cellular longevity*, 2023, 4555609. <https://doi.org/10.1155/2023/4555609>
2. Hu, Q., Chen, Y., Deng, X., Li, Y., Ma, X., Zeng, J., & Zhao, Y. (2023). Diabetic nephropathy: Focusing on pathological signals, clinical treatment, and dietary regulation. *Biomedecine & pharmacotherapie*, 159, 114252. <https://doi.org/10.1016/j.biopha.2023.114252>
3. Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B. B., Stein, C., Basit, A., Chan, J. C. N., Mbanya, J. C., Pavkov, M. E., Ramachandaran, A., Wild, S. H., James, S., Herman, W. H., Zhang, P., Bommer, C., Kuo, S., Boyko, E. J., & Magliano, D. J. (2022). IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes research and clinical practice*, 183, 109119. <https://doi.org/10.1016/j.diabres.2021.109119>.
4. Abhinav, R. P., Williams, J., Livingston, P., Anjana, R. M., & Mohan, V. (2020). Burden of diabetes and oral cancer in India. *Journal of diabetes and its complications*, 34(11), 107670. <https://doi.org/10.1016/j.jdiacomp.2020.107670>.
5. Dewark, S., Murthy, C., & Mohan, V. (2020). Diabetes in India-Challenges, Solutions, and Opportunities.
6. Huang, T. S., Wu, T., Wu, Y. D., Li, X. H., Tan, J., Shen, C. H., Xiong, S. J., Feng, Z. Q., Gao, S. F., Li, H., & Cai, W. B. (2023). Long-term statins administration exacerbates diabetic nephropathy via ectopic fat deposition in diabetic mice. *Nature communications*, 14(1), 390. <https://doi.org/10.1038/s41467-023-35944-z>.
7. DeFronzo, R. A., Reeves, W. B., & Awad, A. S. (2021). Pathophysiology of diabetic kidney disease: impact of SGLT2 inhibitors. *Nature reviews. Nephrology*, 17(5), 319–334. <https://doi.org/10.1038/s41581-021-00393-8>.
8. Giglio, R. V., Patti, A. M., Rizvi, A. A., Stoian, A. P., Ciaccio, M., Papanas, N., Janez, A., Sonmez, A., Banach, M., Sahebkar, A., & Rizzo, M. (2023). Advances in the Pharmacological Management of Diabetic Nephropathy: A 2022 International Update. *Biomedicines*, 11(2), 291. <https://doi.org/10.3390/biomedicines11020291>.
9. Rungratanawanich, W., Qu, Y., Wang, X., Essa, M. M., & Song, B. J. (2021). Advanced glycation end products (AGEs) and other adducts in aging-related diseases and alcohol-mediated tissue injury. *Experimental & molecular medicine*, 53(2), 168–188. <https://doi.org/10.1038/s12276-021-00561-7>.

10. Gkogkolou, P., & Böhm, M. (2012). Advanced glycation end products: Key players in skin aging? *Dermato-endocrinology*, 4(3), 259–270. <https://doi.org/10.4161/derm.22028>.
11. Parwani, K., Patel, F., Bhagwat, P., Dilip, H., Patel, D., Thiruvencatam, V., & Mandal, P. (2021). Swertiamarin mitigates nephropathy in high-fat diet/streptozotocin-induced diabetic rats by inhibiting the formation of advanced glycation end products. *Archives of physiology and biochemistry*, 1–19. Advance online publication. <https://doi.org/10.1080/13813455.2021.1987478>.
12. Marín, L., Gutiérrez-Del-Río, I., Yagüe, P., Manteca, Á., Villar, C. J., & Lombó, F. (2017). *De Novo* Biosynthesis of Apigenin, Luteolin, and Eriodictyol in the Actinomycete *Streptomyces albus* and Production Improvement by Feeding and Spore Conditioning. *Frontiers in microbiology*, 8, 921. <https://doi.org/10.3389/fmicb.2017.00921>.
13. Ley, J. P., Krammer, G., Reinders, G., Gatfield, I. L., & Bertram, H. J. (2005). Evaluation of bitter masking flavanones from Herba Santa (*Eriodictyon californicum* (H. and A.) Torr., Hydrophyllaceae). *Journal of agricultural and food chemistry*, 53(15), 6061–6066. <https://doi.org/10.1021/jf0505170>.
14. Manthey, J. A., Grohmann, K., & Guthrie, N. (2001). Biological properties of citrus flavonoids pertaining to cancer and inflammation. *Current medicinal chemistry*, 8(2), 135–153. <https://doi.org/10.2174/0929867013373723>.
15. Cao, G., Sofic, E., & Prior, R. L. (1997). Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free radical biology & medicine*, 22(5), 749–760. [https://doi.org/10.1016/s0891-5849\(96\)00351-6](https://doi.org/10.1016/s0891-5849(96)00351-6).
16. Ferriola, P. C., Cody, V., & Middleton, E., Jr (1989). Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. *Biochemical pharmacology*, 38(10), 1617–1624. [https://doi.org/10.1016/0006-2952\(89\)90309-2](https://doi.org/10.1016/0006-2952(89)90309-2).
17. Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Révész, C., Chap, H., & Payrastre, B. (1997). Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. *Biochemical pharmacology*, 53(11), 1649–1657. [https://doi.org/10.1016/s0006-2952\(97\)82453-7](https://doi.org/10.1016/s0006-2952(97)82453-7).
18. Mutoh, M., Takahashi, M., Fukuda, K., Komatsu, H., Enya, T., Matsushima-Hibiya, Y., Mutoh, H., Sugimura, T., & Wakabayashi, K. (2000). Suppression by flavonoids of cyclooxygenase-2 promoter-dependent transcriptional activity in colon cancer cells: structure-activity relationship. *Japanese journal of cancer research: Gann*, 91(7), 686–691. <https://doi.org/10.1111/j.1349-7006.2000.tb01000.x>.
19. Ko, W. C., Shih, C. M., Lai, Y. H., Chen, J. H., & Huang, H. L. (2004). Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure-activity relationships. *Biochemical pharmacology*, 68(10), 2087–2094. <https://doi.org/10.1016/j.bcp.2004.06.030>.
20. Kilanowska, A., & Ziolkowska, A. (2020). Role of Phosphodiesterase in the Biology and Pathology of Diabetes. *International journal of molecular sciences*, 21(21), 8244. <https://doi.org/10.3390/ijms21218244>.
21. Park, S. J., Lee, Y. H., Lee, K. H., & Kim, T. J. (2013). Effect of eriodictyol on the development of atopic dermatitis-like lesions in ICR mice. *Biological & pharmaceutical bulletin*, 36(8), 1375–1379. <https://doi.org/10.1248/bpb.b13-00296>.
22. Lee, E. R., Kim, J. H., Kang, Y. J., & Cho, S. G. (2007). The anti-apoptotic and anti-oxidant effect of eriodictyol on UV-induced apoptosis in keratinocytes. *Biological & pharmaceutical bulletin*, 30(1), 32–37. <https://doi.org/10.1248/bpb.30.32>.

23. Liu, Y., & Yan, X. (2019). Eriodictyol inhibits survival and inflammatory responses and promotes apoptosis in rheumatoid arthritis fibroblast-like synoviocytes through AKT/FOXO1 signaling. *Journal of cellular biochemistry*, 120(9), 14628–14635. <https://doi.org/10.1002/jcb.28724>.
24. Bucolo, C., Leggio, G. M., Drago, F., & Salomone, S. (2012). Eriodictyol prevents early retinal and plasma abnormalities in streptozotocin-induced diabetic rats. *Biochemical pharmacology*, 84(1), 88–92. <https://doi.org/10.1016/j.bcp.2012.03.019>.
25. Hameed, A., Hafizur, R. M., Hussain, N., Raza, S. A., Rehman, M., Ashraf, S., Ul-Haq, Z., Khan, F., Abbas, G., & Choudhary, M. I. (2018). Eriodictyol stimulates insulin secretion through cAMP/PKA signaling pathway in mice islets. *European journal of pharmacology*, 820, 245–255. <https://doi.org/10.1016/j.ejphar.2017.12.015>.
26. Bai, J., Wang, Y., Zhu, X., & Shi, J. (2019). Eriodictyol inhibits high glucose-induced extracellular matrix accumulation, oxidative stress, and inflammation in human glomerular mesangial cells. *Phytotherapy research: PTR*, 33(10), 2775–2782. <https://doi.org/10.1002/ptr.6463>.
27. Mariyappan, P., Kalaiyarasu, T., & Manju, V. (2017). Effect of eriodictyol on preneoplastic lesions, oxidative stress and bacterial enzymes in 1,2-dimethyl hydrazine-induced colon carcinogenesis. *Toxicology research*, 6(5), 678–692. <https://doi.org/10.1039/c7tx00074j>.
28. Wang, Z., Lan, Y., Chen, M., Wen, C., Hu, Y., Liu, Z., & Ye, L. (2017). Eriodictyol, Not Its Glucuronide Metabolites, Attenuates Acetaminophen-Induced Hepatotoxicity. *Molecular pharmaceutics*, 14(9), 2937–2951. <https://doi.org/10.1021/acs.molpharmaceut.7b00345>.
29. Ferreira, E.deO., Fernandes, M. Y., Lima, N. M., Neves, K. R., Carmo, M. R., Lima, F. A., Fonteles, A. A., Menezes, A. P., & Andrade, G. M. (2016). Neuroinflammatory response to experimental stroke is inhibited by eriodictyol. *Behavioural brain research*, 312, 321–332. <https://doi.org/10.1016/j.bbr.2016.06.046>.
30. Mao, Z., Gan, C., Zhu, J., Ma, N., Wu, L., Wang, L., & Wang, X. (2017). Anti-atherosclerotic activities of flavonoids from the flowers of *Helichrysum arenarium* L. MOENCH through the pathway of anti-inflammation. *Bioorganic & medicinal chemistry letters*, 27(12), 2812–2817. <https://doi.org/10.1016/j.bmcl.2017.04.076>.
31. Kuhad, A., & Chopra, K. (2009). Attenuation of diabetic nephropathy by tocotrienol: involvement of NFκB signaling pathway. *Life sciences*, 84(9-10), 296–301. <https://doi.org/10.1016/j.lfs.2008.12.014>.
32. Kadian, S., Mahadevan, N., & Balakumar, P. (2013). Differential effects of low-dose fenofibrate treatment in diabetic rats with early onset nephropathy and established nephropathy. *European journal of pharmacology*, 698(1-3), 388–396. <https://doi.org/10.1016/j.ejphar.2012.10.012>.
33. Soetikno, V., Sari, F. R., Veeraveedu, P. T., Thandavarayan, R. A., Harima, M., Sukumaran, V., Lakshmanan, A. P., Suzuki, K., Kawachi, H., & Watanabe, K. (2011). Curcumin ameliorates macrophage infiltration by inhibiting NF-κB activation and proinflammatory cytokines in streptozotocin induced-diabetic nephropathy. *Nutrition & metabolism*, 8(1), 35. <https://doi.org/10.1186/1743-7075-8-35>.
34. Kundu, A., Dey, P., Sarkar, P., Karmakar, S., Tae, I. H., Kim, K. S., Park, J. H., Lee, S. H., Lee, B. M., Renthlei, L., Puia, Z., & Kim, H. S. (2020). Protective effects of *Croton hookeri* on streptozotocin-induced diabetic nephropathy. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 135, 110873. <https://doi.org/10.1016/j.fct.2019.110873>.
35. Xie, L., Zhai, R., Chen, T., Gao, C., Xue, R., Wang, N., Wang, J., Xu, Y., & Gui, D.

- (2020). Panax Notoginseng Ameliorates Podocyte EMT by Targeting the Wnt/ β -Catenin Signaling Pathway in STZ-Induced Diabetic Rats. *Drug design, development and therapy*, 14, 527–538. <https://doi.org/10.2147/DDDT.S235491>.
36. Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical biochemistry*, 95(2), 351–358. [https://doi.org/10.1016/0003-2697\(79\)90738-3](https://doi.org/10.1016/0003-2697(79)90738-3).
37. ELLMAN G. L. (1959). Tissue sulfhydryl groups. *Archives of biochemistry and biophysics*, 82(1), 70–77. [https://doi.org/10.1016/0003-9861\(59\)90090-6](https://doi.org/10.1016/0003-9861(59)90090-6).
38. Claiborne, A., 1985. Catalase activity. In: Greenwald, R.A (Ed.), *Handbook of Methods for Oxygen Radical Research*. CRC Press Inc., pp. 283–284.
39. Sastry, K. V., Moudgal, R. P., Mohan, J., Tyagi, J. S., & Rao, G. S. (2002). Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. *Analytical biochemistry*, 306(1), 79–82. <https://doi.org/10.1006/abio.2002.5676>.
40. Suzuki, K., 2000. Measurement of Mn-SOD and Cu, Zn-SOD. In: Taniguchi, N., Gutteridge, M.C.J. (Eds.), *Experimental Protocols for Reactive Oxygen and Nitrogen Species*. Oxford University Press, Oxford, N.Y., pp. 91–95.
41. Szkudelski T. (2012). Streptozotocin-nicotinamide-induced diabetes in the rat. Characteristics of the experimental model. *Experimental biology and medicine (Maywood, N.J.)*, 237(5), 481–490. <https://doi.org/10.1258/ebm.2012.011372>.
42. Eleazu, C. O., Eleazu, K. C., Chukwuma, S., & Essien, U. N. (2013). Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. *Journal of diabetes and metabolic disorders*, 12(1), 60. <https://doi.org/10.1186/2251-6581-12-60>.
43. Kaikini, A. A., Dhodi, D., Muke, S., Peshattiwar, V., Bagle, S., Korde, A., Sarnaik, J., Kadwad, V., Sachdev, S., & Sathaye, S. (2020). Standardization of type 1 and type 2 diabetic nephropathy models in rats: Assessment and characterization of metabolic features and renal injury. *Journal of pharmacy & bioallied sciences*, 12(3), 295–307. https://doi.org/10.4103/jpbs.JPBS_239_19.
44. Palygin, O., Spires, D., Levchenko, V., Bohovyk, R., Fedoriuk, M., Klemens, C. A., Sykes, O., Bukowy, J. D., Cowley, A. W., Jr, Lazar, J., Ilatovskaya, D. V., & Staruschenko, A. (2019). Progression of diabetic kidney disease in T2DN rats. *American journal of physiology. Renal physiology*, 317(6), F1450–F1461. <https://doi.org/10.1152/ajprenal.00246.2019>.
45. Amin, A., Bajbouj, K., Koch, A., Gandesiri, M., & Schneider-Stock, R. (2015). Defective autophagosome formation in p53-null colorectal cancer reinforces crocin-induced apoptosis. *International journal of molecular sciences*, 16(1), 1544–1561. <https://doi.org/10.3390/ijms16011544>.
46. Raina, R., Chauvin, A., Chakraborty, R., Nair, N., Shah, H., Krishnappa, V., & Kusumi, K. (2020). The Role of Endothelin and Endothelin Antagonists in Chronic Kidney Disease. *Kidney diseases (Basel, Switzerland)*, 6(1), 22–34. <https://doi.org/10.1159/000504623>.