Original Article

Nephroprotective Activity Of Hesperidin Against Streptazocine - Nicotinamide Induced Diabetic Nephropathy In Rats And Its Role On Nitric Oxide Pathway

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Abstract

Aim: The effect of Hesperidin on nitric oxide pathway in Diabetic Nephropathy (DN) rat model.**Method:**In *In vitro* studies the effect of Hesperidin on high glucose-induced damage of human renal glomerular endothelial cells was studied by MTT assay. Endothelial Cell Permeability and Nitric Oxide Scavenging Activity of Hesperidin was performed by clearance of albumin across endothelial monolayers and Griess reaction.*In vivo*studies examined the effect of Hesperidin on the nitric oxide pathway in rat diabetic nephropathy model with a single dose of Streptazocine (STZ) (55mg/kg b.w, i.p) prior to Nicotinamide (NCT) (100 mg/kg b.w, i.p). Once diabetes mellitus was induced, animal groups were treated with Captopril, L-NAME, and L-Arginine, Hesperidin – low and high doses & Hesperidin high dose + captopril from 1st week to 9th week of the study. At the end of the study, blood and 24hr urine samples were collected from all the animals for the estimation of serum and urine biochemical parameters including NO_3^-/NO_2^- levels. All the animals were sacrificed and kidneys were isolated for the estimation of Lipid peroxidation, total renal collagen content, and kidney histological studies. **Results:**Hesperidin *in vitro* studies showed a reduction in high glucose-induced HRGEC cell apoptosis and percentage of BSA clearance.IC50 value of Hesperidin was found to be 4.86 µg/ml.In *in vivo* study,Hesperidin showed significant protective activity against STZ – NCT-produced renal damage. A high dose (100mg/kg b.w.) of Hesperidin and Hesperidin in combination with captopril was found more effective.**Conclusion**: All of the findings show that hesperidin is a viable medication for DN prevention and has a protective effect against STZ-induced rats.

Keywords: Diabetic nephropathy, diabetes mellitus, nitric oxide pathway, hesperidin, renal disease, and Streptazocine.

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic illnesses characterized by hyperglycaemia caused by decreased insulin production, dysregulated biological activity, or both (1). Diabetic nephropathy (DN) is a primary cause of kidney damage in individuals, affecting over 40% of type I and type II diabetics. Diabetes mellitus (DM) and hypertension are now thought to be the most prevalent causes of end-stage renal failure (ESRD) (2).

Endothelial dysfunction is critical in the development and progression of diabetic vascular disorders including nephropathy. Nitric oxide (NO, a gaseous lipophilic molecule) acts as a free radical. NO is a kind of paracrine mediator that is produced by a set of enzymes called Nitric oxide synthases (NOSs) that convert L-Arginine to L-Citrulline& NO. Nitric oxide synthases (NOS) are classified into three types: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) (3). NO synthesized by eNOS in endothelial cells is important in many of these endothelial functions. Diabetes modifies renal structure and function via abnormalities in nitric oxide (NO) production. (4).

Based on the available experimental data early nephropathy in diabetes is linked to increased intrarenal NO generation, which is predominantly mediated by constitutively released NO (eNOS and nNOS). The increased NO generation may be a factor in

early diabetic nephropathy's hyperfiltration and microalbuminuria. On the other hand, the majority of research shows that advanced nephropathy is connected to increasing NO shortage and causes severe proteinuria, deteriorating renal function, and hypertension. To cure diabetic nephropathy, it may be possible to target the nitric oxide pathway (5). Therefore, the goal of the current study is to assess Hesperidin's ability to protect against diabetic nephropathy and its impact on the nitric oxide pathway.

Hesperidin is a common and affordable plant flavonoid produced mostly from citrus species such as sweet oranges and lemons. Based on the literature, Hesperidin showed Anti-Diabetic Activity. Hesperetin (Hst) is a hesperidin aglycone. Anti-hyperlipidemic, anti-inflammatory, anti-oxidative, anti-hypertensive, and anti-atherogenic activities have been described for hesperidin and hesperetin (6-8). Hesperidin has been shown to alleviate diabetic retinopathy symptoms (9,10), diabetic neuropathy (11), and nephropathy (12).

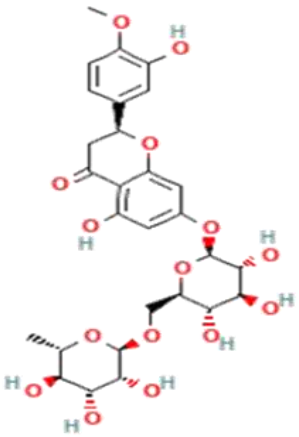


Fig 1: Chemical Structure of Hesperidin

IN - VITRO STUDIES

Materials and Methods

MTT Assay/ Cell Proliferation Assay

3 – [4, 5 – dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay is used to assess the cell viability, proliferation, & cytotoxicity by measuring cellular metabolic activity. The MTT Assay was used to assess the cell viability of HRGECs to rule out the likelihood of cell damage caused by high glucose exposure to Hesperidin [13]

Methodology

The cells were grown for 48 hours at a density of 8 X 10^3 cells per well in medium containing normal glucose (Control) and 30 mM glucose (High glucose; HG). The HRGECs were exposed to 50 M of hesperidin for 12 hours before being cultivated for 48 hours (HG + Hesperidin) in medium containing 30 mM glucose. The L-NAME, a NO inhibitor, and 50 M of (Hesperidin) were applied to the HRGECs for 12 hours, and they were subsequently cultivated for 48 hours in media containing 30 mM glucose (HG + (hesperidin) + L-NAME). Each well's ultimate volume of cell culture media was 200 µL. Each well received a dose of HRGECs culture medium (Procell, CM-H061) containing 0.5 mg/ml MTT. After 4 hours at 37 °C, the culture medium was withdrawn, and 150µL of DMSO (Beyotime, ST038) solution was added. Finally, a microplate reader was used to measure the optical density at 570 nm (BIOTEK, ELX-800).

Endothelial Cell Permeability Assay

Methodology

Albumin diffusion across endothelial monolayers was evaluated to detect endothelial permeability. Briefly, passage 2 of HRGECs were seeded on cell culture inserts (3.0 m) covered with type I collagen, and the inserts were subsequently cultured at 37^0 C in a 5% Carbon dioxide (CO_2)/95% air incubator. Every day, new media was added to the inserts and lower wells. Phase-contrast microscopy was used to check the integrity of HRGEC monolayers grown to confluence on membranes. Cells were grown on inserts for 4 days before being cultured for 48 hours with glucose (Control) or 30mM glucose (High glucose; HG).The HRGECs were exposed to 50 M of Hesperidin for 12 hours before being cultivated for 48 hours (HG + Hesperidin) in a medium containing 30 mM glucose. HG + (Hesperidin) + L-NAME) to both inserts and lower wells were incubated for 1 hour after the HRGECs were treated with 100 M L-NAME (a NO inhibitor) and 50µM Hesperidin for 12 h. Thereafter, the HRGECs were grown in a medium containing 30 mM glucose for 48 h. 750µL of phosphate buffer saline and 150µL of trypan

blue-labelled albumin (0.035% Trypan blue and 0.8% bovine serum albumin (BSA) fraction V) were added to each lower well after washing with ice-cold PBS to halt the drug-enzyme reaction. Similar hydrostatic pressures on either side of the HRGECs monolayer were guaranteed by these volumes. In a 5% CO_2 incubator, the incubation process was maintained for 30 min at 37^0 C. After the incubation period, the inserts were carefully removed, and each lower well's media was well mixed. A 50µL sample from the insert and lower well was taken to measure the absorbance at 590 nm with an ultraviolet (UV) spectrophotometer to assess how much albumin had penetrated the cell monolayer. The percentage clearance of BSA from the insert to the lower well was used to measure the permeability of the HRGECs monolayer [14].

Nitric oxide scavenging activity

To assess the scavenging ability of natural biomolecules against nitric oxide radicals, a nitric oxide radical scavenging technique was used. The Griess Reaction was used to detect nitric oxide produced by sodium nitroprusside [15 – 16]

Methodology

Nitric oxide is created in biological tissues by nitric oxide synthases turning arginine into citrulline. The hesperidin sample was added to 0.5 ml of phosphate buffer saline (pH 7.4) together with 2 ml of sodium nitroprusside (10 mM) at varied concentrations (2 - 10µg/ml) to perform nitric oxide radical scavenging activities. The mixture was then incubated at 25°C, and 0.5 ml of the resulting solution was then removed and combined with 0.5 ml of the Griess reagent [1.0 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthyl ethylenediamine dichloride (0.1% w/v)]] then the absorbance at 546 nm was measured.

The following formula was used to determine scavenging activity:

$$\textbf{Formulae: } \% \text{ scavenging activity} = \frac{A_{Control} - A_{Test}}{A_{Control}} \times 100$$

IN - VIVO STUDIES

Materials and Methods

Chemicals

Hesperidin (ASH2609), captopril (211875-1GM), L-NAME (29299090), and L-Arginine (29224990) were procured from Sisco research laboratories Pvt.Ltd, Mumbai. Streptazocine (STZ) (29420090) and Nicotinamide (NCT) (29362920) were obtained by sigma Aldrich Pvt. Ltd, USA. All other compounds used in the study were of analytical grade

Animals and Diet

Male Sprague Dawley rats weighing about 180-200g and female swiss albino mice weighing about 10 – 20g were procured from Vyas labs, Hyderabad, and acclimatized in an animal house for one week at a temperature of 29^0c to 32^0c with 60 – 80% humidity, 12h dark & light cycle. Regular commercial diet was supplied from VRK Nutritional supplies, Hyderabad.

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), GITAM School of Pharmacy with approval no: IAEC/GIP-128/PM – R.S/Approved/2/2021.

Oral acute toxicity study

The acute toxicity study was performed based on OECD guidelines 423. Three female mice of weight 10-20 g fasted overnight before the administration of Hesperidin orally with a dose of 2000mg/kg. b.w. Animals were observed for signs of mortality and toxicity for 14 days. Animals were kept under observation for the changes that occurred in skin and fur, eyes and mucous membrane, respiratory, autonomic, circulatory, and central nervous system, behavioural changes, tremors, convulsions, salivation, diarrhoea, sleep, and coma. From the maximum dose, 1/20th and 40th dose was selected for further in-vivo studies.

EXPERIMENTAL METHODOLOGY

Induction of diabetic nephropathy

After 1 week of the acclimation phase, the weight of the male Sprague Dawley rats was measured. Rats were allowed to fast for 10hours before the induction of diabetes. Each Rat received a single dose of nicotinamide (100 mg/Kg B.W, I.p) before Streptazocine injection (55mg/kg B.W, I.p). After 6hours of STZ injection, animals were given 5% glucose for 24 h to prevent

streptozotocin-induced hypoglycaemia mortality. The fasting blood glucose levels were measured on 3rd day after the STZ injection. Rats having fasting blood glucose of more than 200mg/dL were confirmed as diabetic.

Hesperidin suspension was prepared in 1% sodium carboxy methyl cellulose (CMC). Standard drug (captopril), L-NAME, and L-Arginine were dissolved in Distilled water.

Experimental Design
Forty-eight Animals were divided into Eight groups each containing six animals. Treatment is as follows:

Group1: Normal Control: Animals were treated with 1% Sodium CMC, orally for 9 weeks;

Group 2: Disease Control: A single Dose of 55 mg/kg b.w of STZ I.p. before 100 mg/kg b.w, NCT I. p induces diabetes mellitus (DM);

Group 3: Standard Drug: DM rats were treated with a standard drug(captopril) at a dose of 50mg/kg, b.w. p.o from 1st week to 9th week;

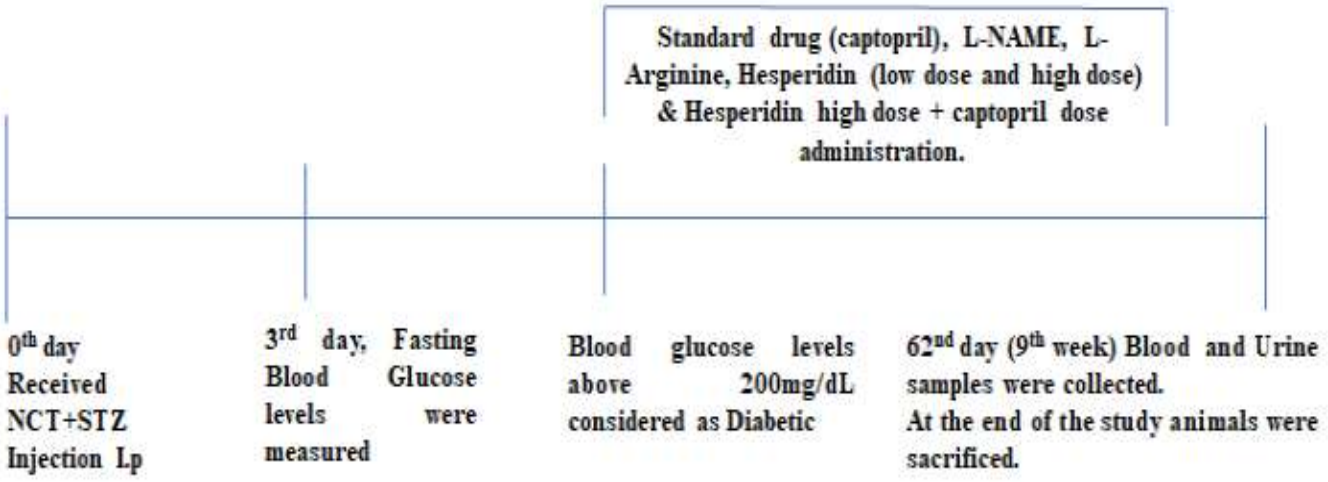
Group 4: L-Name: DM rats were treated with L-NAME at a dose of 100mg/kg b.w orally from 1st week to 9th week;

Group 5: L-Arginine: DM rats were treated with L-Arginine at a dose of 1g/kg b.w orally from 1st week to 9th week;

Group 6: Hesperidin Low Dose: DM rats were treated with Hesperidin low dose at a dose of 50mg/kg, b.w. p.o from 1st week to 9th week;

Group 7: Hesperidin High Dose: DM rats were treated with Hesperidin high dose at a dose of 100mg/kg, b.w. p.o from 1st week to 9th week;

Group 8: Hesperidin + Standard Drug: DM rats were treated with Hesperidin (high dose) + standard drug from 1st week to 9th week.



At the end of the 9th week, Blood samples were collected by a retro-orbital puncture for estimation of serum creatinine, Blood urea nitrogen, blood glucose levels, and Serum NO_3^-/NO_2^- levels. 24 h urine samples were collected using a metabolic cage for estimation of urine albumin, total protein, and urinary NO_3^-/NO_2^-levels. At the end of the study, all the animals were sacrificed under high doses of anaesthesia, and kidneys were dissected and used for lipid peroxidation, NO_3^-/NO_2^-levels, and histological studies.

PARAMETERS ESTIMATED

Body weight
All the animals were weighed every week using a digital balance.

Measurement of Serum and Urine Biochemical Parameters
Jaffe's method was used to measure serum creatinine [17]. Blood urea nitrogen, albumin, and total protein were estimated by kits of Excel Diagnostics Pvt.Ltd., Hyderabad, India.

Nitrite (NO_3^-) and Nitrate (NO_2^-) Assay
This assay is used to determine the levels of NO_3^-/NO_2^-in serum, urine &Total kidney quantitatively by colorimetry. It is estimated by using the Kamiya Biomedical company kit procedure.

Kidney Fractional Weight
After the euthanization of the animals, immediately kidneys were dissected out; washed in saline & blotted on a filter paper, and weighed on a digital balance. Kidney fractional weight was calculated by the following

$$\textbf{Formula} = \frac{\text{Kidney weight}}{\text{Total body weight}} \times 100$$

Lipid peroxidation

Kidney homogenate
10% of the kidney homogenate from one isolated kidney was prepared by separate homogenization in an ice-cold buffer with 0.1M pH - 7.4. The homogenate was centrifuged for 20 minutes at 10,000 rpm and the supernatant was collected.

Procedure
0.5 ml of kidney homogenate, 3 ml phosphoric acid (1%), and 1 ml of Thio barbituric acid (0.6%) were added in a centrifuge tube and then the mixture was kept for 45min in a boiling water bath. After cooling the mixture, 4ml of n-butanol was added, vortexed for 1min, then centrifuged at 20000 rpm for 20 mins. The layer was collected in a separate fresh tube and its absorbance was measured at 532 nm using a spectrophotometer (18).

Calculation

MDA (mmol/gr tissue) = absorbance/1.56 X 105

Histopathology
The other dissected kidneys from each rat were washed in saline water, fixed in 10% formalin for 48 hours, dried in ethanol (50-100%), and then embedded in paraffin wax. Using a microtome, the slices were cut at a thickness of 5 m, and they were then stained for microscopic analysis using hematoxyline and eosin dye. Under 400x slides were read.

Statistical analysis
Analysis was done using the software graph pad prism, version -8.0. all data presented as mean ± SEM n=6. $^{###}p<0.001$ when compared to the normal control group; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 when compared to the Disease control group, evaluated by using one-way ANOVA test followed by Tukey's multiple comparison test.

RESULTS

In Vitro Study

1. **MTT assay/ cell proliferation assay**

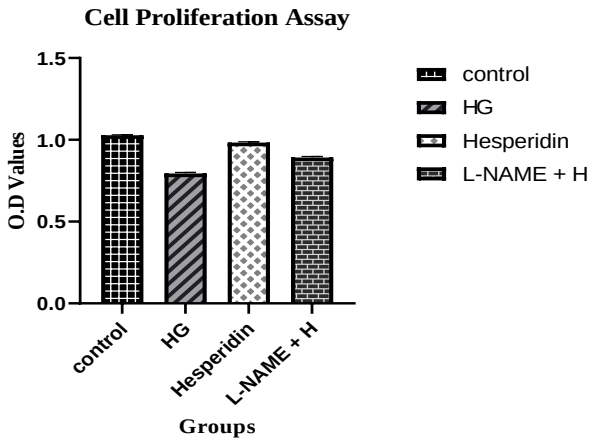


Fig 2: Cell Proliferation Assay

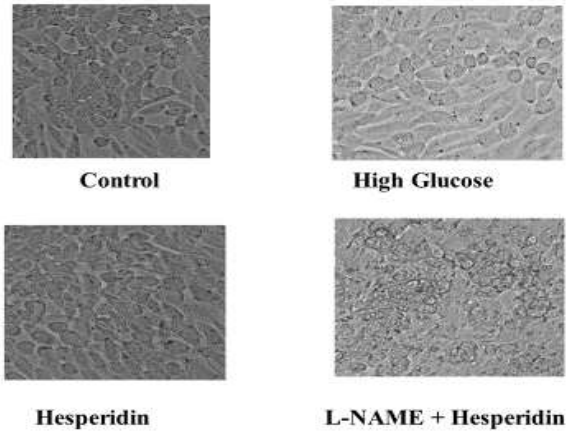


Fig 3: Photographs of Cell Proliferation Assay.

2. Endothelial Cell Permeability Assay

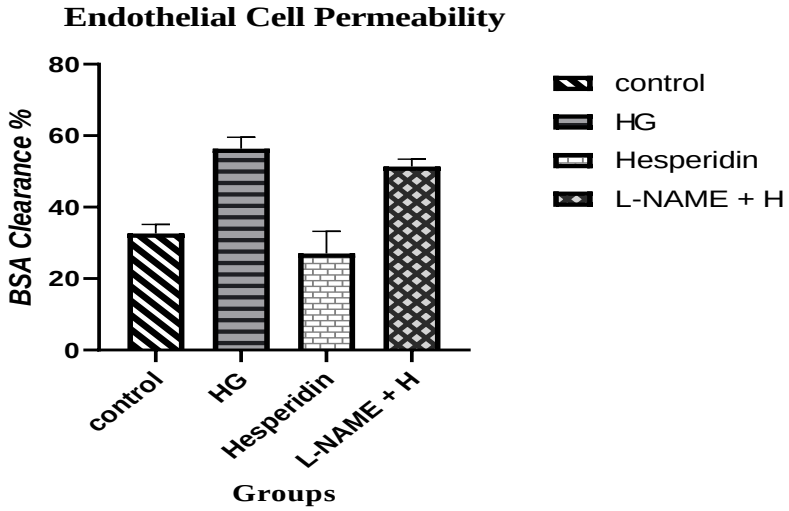


Fig 4: Endothelial Cell Permeability Assay

3. Nitric oxide scavenging activity

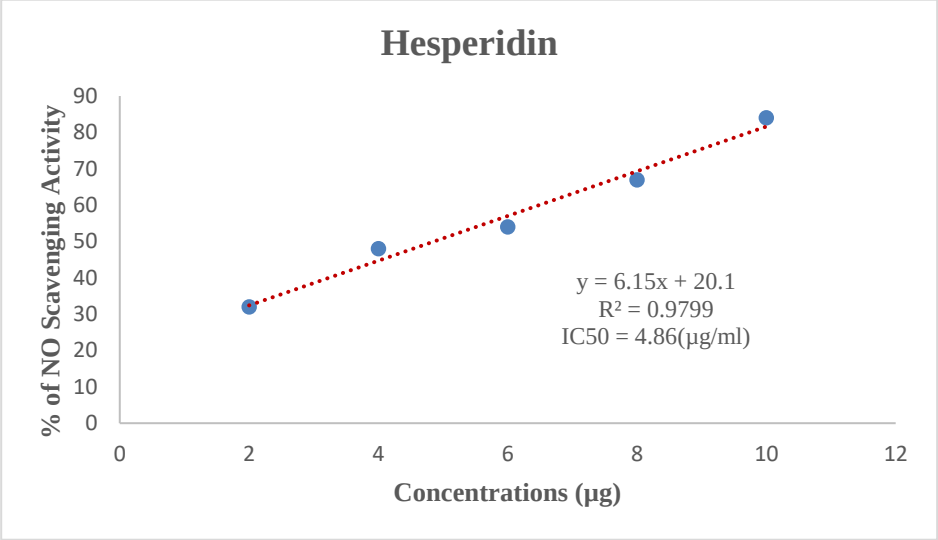


Fig 5: Nitric oxide scavenging Assay

In Vivo Study

1. Body weight

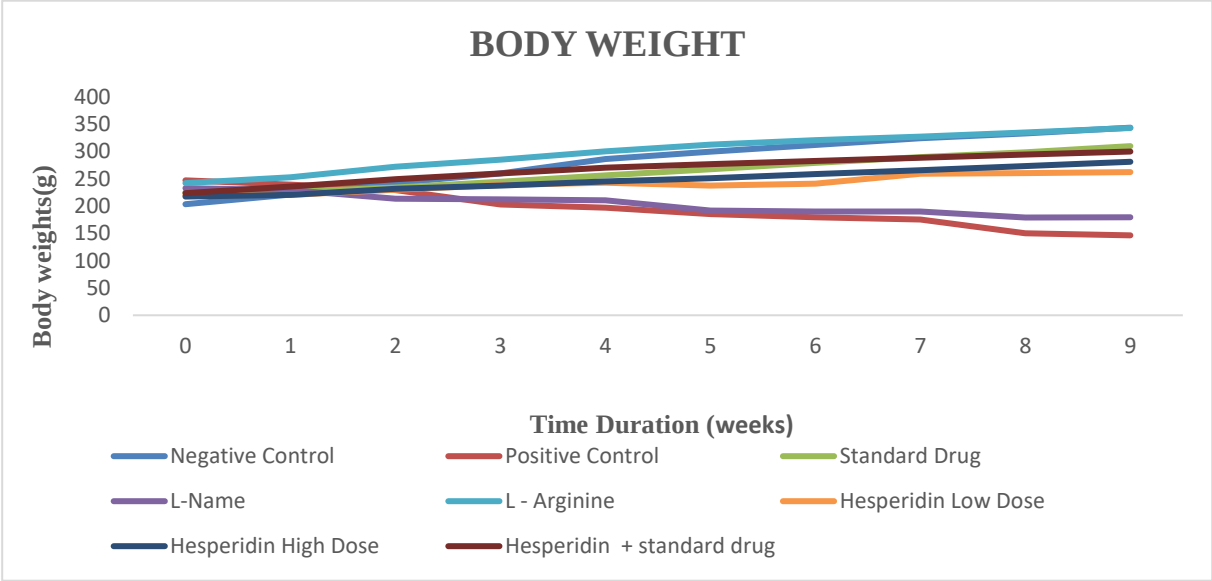


Fig 6: Body weight of Sprague Dawley rats during 9 weeks of study

Table No.1: Fasting Blood glucose levels (mg/dL) on the 3rdand 62nd days of the study

Groups	3rd day	62nd day
Normal Control	130±6.1	114±3.1
Disease Control	326±32	496±16####
Captopril	345±50	318±61*
L-Name	387±48	275±63*
L - Arginine	409±55	190±26***
Hesperidin 50mg/kg	383±22	242±37**
Hesperidin 100mg/kg	335±12	176±26***
Hesperidin 100mg/kg + captopril	320±25	118±3.6***

The data above were presented as mean ± SEM n=6. ### p<0.001 when compared to the normal control group; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 when compared to the Disease control group, as determined by using one–way ANOVA followed by Tukey's multiple comparisons tests

Table No.2: Estimation of Blood urea nitrogen, serum creatinine, serum nitrite/ nitrate levels

Groups	Blood urea nitrogen (mg/dl)	Serum creatinine (mg/dl)	Serum nitrate/nitrite levels (µM)
	62nd day	**62nd day**	**62nd day**
Normal Control	15±0.6	0.3±0.06	30±2.03
Disease Control	29±2.9####	0.7±0.03##	13±1.7####
Captopril	18±0.3**	0.26±0.01***	23±0.7**
L-Name	27±3.08ns	0.44±0.08ns	14±2.1ns
L - Arginine	13±0.9***	0.41±0.06*	24±1.7***
Hesperidin 50mg/kg	16±0.5**	0.43±0.07*	22±0.6*
Hesperidin 100mg/kg	12±0.3***	0.21±0.01****	25±0.7****
Hesperidin 100mg/kg + Captopril	18±1.9**	0.28±0.02***	24±1.7***

The data above were presented as mean ± SEM n=6. ### p<0.001 when compared to the normal control group; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 when compared to the Disease control group, as determined by using one–way ANOVA followed by Tukey's multiple comparisons tests.

Table No.3: Estimation of Urine Albumin, Total Protein, and Urinary nitrate / nitrite Levels

Groups	Albumin (g/dl)	Total protein(g/dl)	Urinary nitrate / nitrite levels (µM)
Normal Control	13±0.7	12±0.9	2.3±0.22
Disease Control	30±1.1####	28±0.88####	1.2±0.008#
Captopril	21±0.61*	18±0.80***	2.07±0.16**
L-Name	26±1.3ns	25±1.4ns	1.42±0.004ns
L - Arginine	19±1.4**	18±1.3***	2.17±0.16**
Hesperidin 50mg/kg	20±1.09*	16±1.5**	2.1±0.17**
Hesperidin 100mg/kg	17±1.2***	12±1.4****	2.6±0.16***
Hesperidin 100mg/kg + Captopril	15±1.2***	12±1.3****	2.2±0.1***

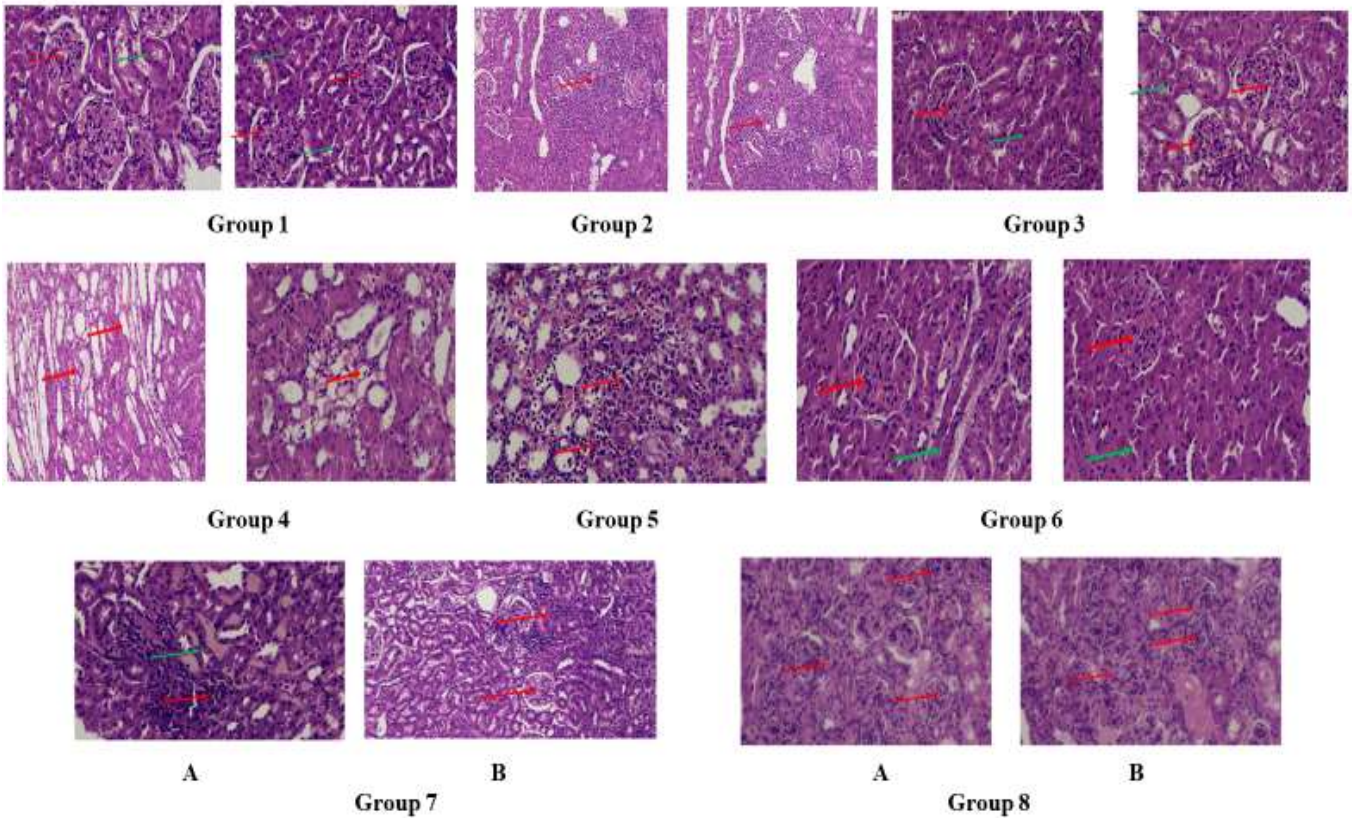
The data above were presented as mean ± SEM n=6. ### p<0.001 when compared to the normal control group; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 when compared to the Disease control group, as determined by using one–way ANOVA followed by Tukey's multiple comparisons tests.

Table No.4: Kidney fractional weight, Lipid peroxidation and Total kidney Nitrite /nitrate levels

Groups	Kidney fractional Weight	MDA levels	Total kidney Nitrite /nitrate levels (µM)
Normal Control	1.9±0.005	69±2.03	2.8±0.02
Disease Control	2.25±0.1	126±11##	1.6±0.09####
Captopril	1.1±0.16***	76±4.2**	2.2±0.1**
L-Name	2.06±0.01ns	113±14ns	1.6±0.2ns
L - Arginine	1.4±0.21*	80±10*	2.3±0.05***
Hesperidin 50mg/kg	1.48±0.21*	77±11**	1.2±0.02*
Hesperidin 100mg/kg	1.03±0.004****	68±1.4***	2.5±0.005***
Hesperidin 100mg/kg + Captopril	1.2±0.19***	65±1.9***	2.1±0.02**

The data above were presented as mean ± SEM n=6. ### p<0.001 when compared to the normal control group; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 when compared to the Disease control group, as determined by using one–way ANOVA followed by Tukey's multiple comparisons tests.

Histological studies



Group 1: Normal Control: Normal morphology of glomerulus, the morphology of tubules in cortex region;

Group 2: Disease control: tubular nephritis, inflammation with infiltration of lymphocytes;

Group 3: Captopril: PAS reaction in the glomeruli, PAS in PCT, and basal laminae of tubules;

Group 4: L-NAME: tubular degeneration and loss of brush border in PCT;

Group 5: L-Arginine: Foci of cystic dilatation;

Group 6: Hesperidin Low Dose: shows distorted and vacuolar degeneration;

Group 7: Hesperidin High dose: shows A) mild congestion with normal hepatic architecture, B) Foci of tubular regeneration and interstitial fibrosis were observed;

Group 8: Hesperidin High dose + captopril: shows A) normal glomerular tuft (g) proximal convoluted tubule and B) distal convoluted tubules

Fig 7: Histology of rat kidneys treated in the Normal control, Disease control, Captopril, L-NAME, L-Arginine, Hesperidin Low and High Doses, Hesperidin + captopril (H&E X400)

DISCUSSION

Hesperidin is a flavonoid that is found in a wide range of various fruits, vegetables, sweet oranges, and lemons. Hesperidin has shown many pharmacological effects that are mostly explained by its antioxidant defence mechanism and reduction of the generation of proinflammatory cytokines (19).

Human renal glomerular endothelial cells (HRGEs) are distinct capillary endothelial cells, and high blood glucose levels promote HRGEs malfunction and death, both are key causes of diabetic nephropathy. The results of the MTT assay showed incubation of HRGECs cells in the High Glucose decreased the cell proliferation compared to the control cells, which was reversed by Hesperidin. With the addition of L-NAME (NO synthesis (NOS) inhibitor) in combination with Hesperidin, the capability of cell proliferation was decreased (Figures 2 & 3).

High glucose levels activate protein kinase C (PKC), a family of kinases required for intracellular signalling. The test hypothesis states that high glucose concentrations in endothelial cells activate PKC, which raises endothelial cell permeability through different PKC isoforms (20). The endothelial barrier maintains a minimal and selective permeability to fluid and chemicals under ordinary physiological settings. High glucose levels cause the endothelium barrier to be disrupted, which increases cell permeability. The results of the endothelial cell permeability assay showed incubation of HRGECs cells in the High Glucose significantly increased in BSA clearance % compared to the control cells, which was reversed by Hesperidin. With the addition of L-NAME with Hesperidin, the capability of cell permeability was further increased (figure 4).

A vital gas for many physiological processes is nitric oxide. However, an excess of reactive oxygen species is a potentially dangerous chemical with free radical capabilities that causes nitrosative stress, which may damage cellular function and change the structure of proteins (21). In *invitro* % of NO scavenging activity of hesperidin was observed. IC50 values of Hesperidin were found to be 4.86µg/ml, (figure 5).

In *In Vivo* study, the diabetic rats showed a significant decrease in body weight compared to normal control whereas captopril, L-Arginine, hesperidin low and high doses, and hesperidin with captopril-treated groups showed a significant increase in the body weight (figure 6).

Creatinine is a good mark for renal kidney function. High Serum creatinine levels suggest ill renal function. In the disease control, Blood urea nitrogen and Serum creatinine were significantly increased when compared to the normal control. In captopril, L-Arginine, hesperidin low and high doses, and hesperidin with captopril treated groups, showed a significant decrease in Blood urea nitrogen and Serum creatinine when compared to disease control and L-NAME treated group showed no significant decrease when compared to disease control.

Albuminuria is a sign of renal damage which means that they have too much albumin in their urine. Total urine protein has been vital in diagnosing and monitoring renal disease. Healthy kidneys do not allow significant amounts of protein to pass through their filters. In the disease control, albumin and total protein showed a significant increase when compared to the normal control. In the captopril, L-Arginine, hesperidin low and high doses and hesperidin with captopril-treated groups showed a significant decrease in the urine albumin and proteins. L-NAME treated group showed no significant decrease when compared to disease control.

The effect of high glucose on the NO levels in serum, urine, and kidneys was assessed by estimating nitrite/nitrate concentration levels. In diabetic rat's nitrite/nitrate concentration levels significantly decreased when compared to normal control and increased with captopril, L-Arginine, hesperidin low and high doses, and hesperidin with captopril-treated groups in serum, urine, and kidney tissue.

Kidney Fractional weight significantly increased in the disease control when compared to the normal control. captopril, L-Arginine, hesperidin low and high doses, and hesperidin with captopril-treated groups, the fractional weight of the kidney decreased significantly but L-NAME treated group showed a significant increase.

Lipid peroxidation is a highly destructive process and leads to many changes in the cell membrane structure and function which leads to cell injury. It is studied indirectly by producing secondary products such as reactive low molecular weight aldehyde-malondialdehyde (MDA). In captopril, L-Arginine, hesperidin low and high doses, and hesperidin with captopril treated groups showed an increase in % reduction of tissue MDA levels compared to the disease control.

In histopathological studies, the normal control of the kidney shows normal morphology of the glomerulus and tubules in the cortex region. The disease control shows Moderate tubular nephritis: Multifocal tubular/interstitial inflammation with

infiltration of lymphocytes. Standard drug (Captopril) shows a strong periodic acid-Schiff (PAS) reaction in the glomeruli, brush border of the proximal convoluted tubules (P) as well as basal laminae of tubules. In L-NAME treated group shows tubular degeneration and loss of brush border were observed in proximal convoluted tubules in the cortex region. In L-Arginine treated group shows Foci of cystic dilatation along with cystic tubular degeneration were observed in collecting ducts in the renal pelvis region. A low dose of Hesperidin shows A) distorted and vacuolar degeneration in most of the tubular epithelial cells of cortical renal tubules and B) the Glomerulus appeared normal. A high dose of Hesperidin shows A) revealed mild congestion but the normal hepatic architecture and B) Foci of tubular regeneration and interstitial fibrosis were observed. In hesperidin, high dose, and standard drug show A) normal glomerular tuft (g) proximal convoluted tubule, and B) distal convoluted tubules (figure 7).

CONCLUSION

A high dose of Hesperidin showed more significant protection against diabetic kidneys by restoring the body weight, kidney weight, serum and urine biochemical parameters, reduced kidney MDA levels, and histological changes when compared to the low dose of Hesperidin. Also, Hesperidin with a combination of captopril showed more significant protection against diabetic kidneys. Nitrite and nitrate in blood have been widely used as an index of endothelial NO synthase activity as routine indirect measures of NO levels. Hesperidin-treated groups with a concentration of nitrite/nitrate levels significantly increased in serum, urine, and kidney tissue levels. The results of the present study showed that Hesperidin attenuated Streptazocine- induced nephrotoxicity by affecting NO levels in the body**.** This research suggests that Hesperidin is a promising molecule for diabetic nephropathy, and could be used as a therapeutic candidate.

CONFLICT OF INTEREST

The authors reported that they had no conflicts of interest.

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