

Isorhamnetin Attenuates Hyperglycemia-Induced Diabetic Nephropathy-Like Alterations in Human Embryonic Kidney Cells (HEK-293): *In vitro* Insights into the Potential Role of FOXO1

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ABSTRACT

Background: Diabetic nephropathy is becoming an alarming concern globally as the percentage of patients is increasing day by day. Although the suffering rate was too high, the existing treatment was very limited and non-targeted. **Objectives:** To overcome this condition, the plant flavonoid Isorhamnetin (ISN) was used in this study to investigate its protective effect on hyperglycemia-mediated diabetic nephropathy. **Materials and Methods:** Cytotoxicity and nephroprotective potency of Isorhamnetin were investigated with the help of a Tetrazolium-based assay. Apoptosis was confirmed by Acridine orange/Ethidium Bromide (AO/EtBr) dual staining and comet assay, where DNA damage was investigated in both methods. Mitochondrial membrane potential was also assessed by the Mito potential kit. Oxidative stress status was analysed by checking the release of Reactive Oxygen Species (ROS) and reduced Glutathione (GSH). The molecular mechanism also involved the expression of FOXO1, which was investigated between the hyperglycemic condition and the isorhamnetin-treated condition. **Results:** Isorhamnetin exhibited an IC_{50} value of 93.50 μ g/mL, but at low dosage (6.25 μ g/mL), it exhibited a great impact on the high glucose-induced HEK 293 cells. A similar trend was continued in dual staining as well as in mitochondrial membrane potential assessment, as isorhamnetin ameliorates the deterioration effect created by the hyperglycemic condition. Isorhamnetin elevated the antioxidant enzyme release (GSH) and pulled down the free radicals generated by high glucose exposure. The FOXO1 gene was downregulated in the hyperglycemic group, which was reversed in the isorhamnetin-treated group. **Conclusion:** Thus, the evidence proved that Isorhamnetin provides beneficial positive impacts on the diabetic nephropathy condition. Since there are very few studies, the *in vivo* investigation further adds depth to molecular knowledge about detailed signaling pathways.

Keywords: Diabetic Nephropathy, End-stage renal disease, Flavonoids, FOXO1 gene, Isorhamnetin.

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INTRODUCTION

Diabetic Nephropathy (DN), also referred to as Diabetic Kidney Disease (DKD), is a chronic and progressive renal complication of diabetes mellitus. The untreated CKD leads to End-Stage Renal Disease (ESRD).¹ DN arises as a progressive complication of diabetes mellitus and remains a significant contributor to

global morbidity and mortality. According to reports from the International Diabetes Federation (IDF), CKD is projected to become the fifth leading cause of global mortality by 2040. Current statistical reports indicate that nearly 850 million individuals worldwide are affected by kidney-related disorders.^{2,3} DN is predominantly associated with long-standing diabetes mellitus, with clinical evidence suggesting that approximately 40% of diabetic patients develop renal complications, including glomerular hyperfiltration and progressive Renal Interstitial Fibrosis (RIF), ultimately leading to functional renal impairment.^{4,5} The burden of diabetic kidney disease is particularly pronounced in Asian populations, where the prevalence of both type 1 and type 2 diabetes continues to rise. Notably, India and China account for a substantial proportion of global cases, reporting



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annual CKD incidence rates of 34.4% and 13.4%, respectively, thereby intensifying both regional and global health demand.⁶ In response to the escalating impact of kidney diseases and other non-communicable conditions, the World Health Organisation (WHO) has set a strategic goal to reduce premature mortality from non-communicable diseases by one-third by 2030, emphasising the urgent need for effective preventive and therapeutic strategies targeting diabetic nephropathy.³

The progression of Diabetic Nephropathy (DN) is strongly influenced by persistent hyperglycaemia and elevated blood pressure.^{7,8} The current medications focus on lowering glucose by α -glucosidase inhibitors and Sodium-Glucose Cotransporter-2 (SGLT2) inhibitors, etc., and antihypertensive medications such as Angiotensin-Converting Enzyme (ACE) inhibitors and Angiotensin Receptor Blockers (ARBs), etc. These are temporary treatments and non-specific. Even at the End-Stage Renal Disease (ESRD), dialysis is the primary supportive therapy, followed by renal replacement or transplantation in severe conditions.⁹ Multifactorial complications persist in kidney-related disease, such as extracellular matrix accumulation in the wall of glomerular and tubulointerstitial, EMT transition, renal fibrosis, podocyte injury and tubular cell death. The causative pathology of DN is still unclear, but based on previous research, it is proven that excessive glucose leads to the enormous release of Reactive Oxygen Species (ROS) followed by inflammation. So, the herbal medicine is the pivotal choice to address this issue seriously, since herbal-based products are very supportive in the health and financial aspects.

Isorhamnetin (ISN), a flavonoid abundant in leaves and fruits of *Hippophae rhamnoides* L., has a modified or improved structure of quercetin. Quercetin is metabolised to ISN in the intestine and liver, with better absorption. Hence, in the pharmacokinetic aspect, ISN is a more preferable compound than quercetin.¹⁰ ISN has tremendous pharmacological benefits, such as a protector of cardiovascular and cerebrovascular diseases, and prevents obesity, together with exerting anti-inflammatory, antioxidant, anti-bacterial, anti-viral, and anti-diabetic effects. Hence, ISN has become a hotspot in recent years in various therapeutic domains.¹¹⁻¹³ Additionally, ISN exhibits anti-hypertensive, anti-thrombus, antiplatelet activity, anti-osteoporosis, hepatoprotective and anti-hypoxia effects.¹⁴ ISN-treated Diabetic mellitus-related research papers manifest that ISN has the capability to maintain glucose homeostasis by the glucose uptake process, and also prove its intensifying radical scavenging ability under the oxidative stress condition.^{13,15} ISN-treated DM suggested that ISN may alleviate the renal injury, but there were very few research papers available in that specific aspect in the *in vitro* model. ISN demonstrated nephroprotective properties in NRK-2E and HepG2 kidney cell lines, accompanied by hydrogen sulphide.¹⁶ and in the diabetic rat model through the NF- κ B signalling pathway.¹⁷ In the present study, FOXO1 gene

expression was prioritized due to its established protective role in diabetic nephropathy models. Previous studies have reported that loss of FOXO1 expression through gene knockdown or silencing promotes apoptosis and renal fibrosis in renal cells. Under hyperglycaemic conditions, increased STAT1 expression has also been associated with cellular injury, whereas activation of FOXO1 was shown to counteract these effects by interacting with STAT1, thereby reducing apoptosis and suppressing Epithelial-Mesenchymal Transition (EMT). The protective effect of podocyte cells by FOXO1 is also reported.^{18,19} These findings highlight FOXO1 as a relevant molecular target for investigating reno-protective interventions under diabetic conditions.

In this context, the nephroprotective potential of Isorhamnetin (ISN) was evaluated in high-glucose-induced Human Embryonic Kidney (HEK293) cells through comprehensive cellular and molecular assessments. Cell viability analysis was performed to determine the protective efficacy and optimal dosage range of ISN under hyperglycaemic conditions. The ability of ISN to prevent apoptosis, preserve mitochondrial membrane integrity, and modulate oxidative stress-related enzyme activities was subsequently examined. In addition, molecular-level analysis was conducted to investigate the regulatory effect of ISN on FOXO1 gene expression, providing mechanistic insight into its nephroprotective action.

MATERIALS AND METHODS

Procurement and maintenance of the Cell line

Human Embryonic Kidney cell line (HEK 293) was purchased from the ATCC. Cells were cultured using DMEM media (Sigma Aldrich, USA) with FBS and antibiotics of standard requirements. Cells were maintained in a CO₂ incubator (NBS Eppendorf, Germany).

Sample procurement and preparation

Isorhamnetin (ISN) was obtained from Sigma Aldrich. 1 mg of ISN was dissolved in 1mL of DMEM media and mixed completely using a cyclomixer. The sample was further filtered by a 0.22 μ m syringe filter and stored at 4°C for further use.

ISN cytotoxicity assessment on HEK 293 cell line

ISN was subjected to cytotoxicity assessment as per the protocol suggested earlier.²⁰ HEK 293 cells were seeded at a 96-well cell culture plate at 5000 cells in each well and incubated to reach the maximum confluency. Media was removed from each well, and the prepared sample was then added to the cells at 70-80% confluency at half the dilutions (100, 50, 25, 12.5, and 6.25 μ g/mL) with 2% DMEM media, in triplicate. The sample exposed cells and untreated cells were incubated for 24 hr. At the end of the experiment, the cells were visualized under phase contrast microscopy (Olympus CKX41 with Optika Pro5 CCD camera) for the adverse effects of cells, such as shrinkage, cell wall

breakage, and detachment. The effects were photographed, and then the cells were subjected to MTT analysis. For MTT analysis, the media with the sample was removed from each well, and 200 μ L of 2% media containing MTT (Sigma, M-5655) was added at 5 mg/mL. The plate was incubated for 2-4 hr at 37°C in the dark. Then, the media with MTT was removed, and the formazan crystals were dissolved using absolute DMSO (Sigma Aldrich, USA) of 200 μ L. The colored solution was then read at 540 nm using a microplate reader (ERBA, Germany). Percentage viability was computed by the following formula,

$$\% \text{ viability} = [\text{Mean OD sample} / \text{Mean OD control}] \times 100$$

Nephroprotective effect of ISN

Cells were seeded at 5000 cells/well in a 96-well cell culture plate and incubated for sufficient growth. The cells were exposed to 50 mM high glucose for 24 hr to induce nephrotoxicity. Then ISN was added at various concentrations such as 1.5, 3.1, 6.25, 12.5 and 25 μ g/mL and incubated for 24 hr. The cells' morphology variations were observed through a phase contrast microscope, then the cells were subjected to the MTT analysis, where cells were exposed to 2-4 hr at 5 mg/mL of MTT solution in the dark. After the incubation period, MTT reagent-containing media were aspirated, and the saturated DMSO was used to solubilize the formazan crystal of the cell wall. Then the colored solution was read at 540 nm by a microplate reader. Percentage viability was calculated using the following formula,

$$\% \text{ viability} = [\text{Mean OD sample} / \text{Mean OD control}] \times 100$$

Dual staining-AO/EtBr assay

Dual staining-Acridine orange/Ethidium bromide (AO/EtBr)-was performed to observe the status of apoptosis. Cells were seeded in 6 6-well cell culture plates at an appropriate count and incubated for 70% confluency. Cells were initially exposed to 50 mM Glucose for 24 hr, considered as the HG group. Induced cells were then treated with ISN, denoted as HG + ISN. Untreated/control and ISN alone-treated groups were also maintained for reference. 100 μ g/mL of AO and EtBr, respectively, were added to the wells and kept for 10 min. The stain was removed, and cells were washed twice with 1X PBS. The fluorescence intensity was observed under a fluorescence microscope (Olympus CKX41 with Optika Pro5 camera).

Comet Assay

Cells were seeded in a 6-well cell culture plate at an appropriate count and incubated for 70% confluency. Cells were initially exposed to 50 mM Glucose for 24 hr, considered as the HG group. Induced cells were then treated with ISN, denoted as HG + ISN. Untreated/control and ISN alone-treated groups were also maintained for reference. After incubation, the cells were harvested using trypsin, washed with fresh medium, and processed for the comet assay.²¹

Fully frosted slides were precoated with 1 mL of 1% Normal-Melting-Point Agarose (NMA; Invitrogen, USA) and kept at 4°C. A cell suspension (1×10^4 cells in 5-30 μ L) was mixed with 10 μ L of low-melting-point agarose (Novex, Invitrogen) and layered onto the NMA-coated slide under a coverslip. Slides were maintained at 4°C for 20 min to allow solidification. A final protective layer of 1% NMA (1 mL) was added, and the mixture was again allowed to set at 4°C for 20 min. Slides were then immersed in cold lysis buffer (12.5 μ M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, containing 1% SDS), supplemented with 10% DMSO and 1% Triton X-100 immediately before use. After 2 hr of lysis at 4°C, slides were equilibrated in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13) for 20 min to enable DNA unwinding. Electrophoresis was performed in the same buffer at 25 V (300 mA) for 20 min using a standard power supply (Power Case, Life Technologies). Following electrophoresis, slides were neutralized in 0.4 M Tris buffer (pH 7.5) for 5 min (three washes), air-dried, and stained with 50 μ L ethidium bromide (20 μ g/mL). Comet images were captured using an Olympus CKX41 inverted epifluorescence microscope equipped with an Optika Pro5 CCD camera. DNA damage parameters were quantified using the Tritek Comet Scoring software, and the results were statistically analysed.

Mito potential assay

Mitochondrial Membrane Potential (MMP) assessment is a pivotal step, as its dissipation leads to a breakdown of cellular homeostasis and triggers the collapse of major intracellular signalling pathways. MMP was analysed through flow cytometry using Muse® MitoPotential Kit. Cells of appropriate count were seeded in a 6-well cell culture plate. At the maximum confluency, the cells were induced with 50 mM Glucose for 24 hr. Then, untreated cells were considered as the control group, the group induced with Glucose and left untreated was considered as the HG group, the induced group treated with ISN, and non-induced cells treated with ISN alone. These groups were maintained for 24 hr. At the end of the experiments, cells were harvested by trypsinization. Then, the collected cells were centrifuged. 1 x assay buffer of 95 μ L was used to resuspend the obtained pellet (Muse™ Mito potential dye (MPD) diluted as 1:1000) and incubated for 20 min at 37°C. Then, 5 μ L of 7-AAD reagent was added and followed by 5 min of RT incubation. FACS protocol was carried out to find the MMP by Muse™ Cell analyser. Increased Mito Potential dye denotes a high membrane potential, while decreased intensity in the fluorescence denotes a depolarized membrane. 7-amino-actinomycin D - a dead cell marker that tends to bind with DNA and explore the state of DNA. Cell population output by the Mito kit as follows,

1. MPD (+) and 7-AAD (+) - Dead cells with depolarized mitochondrial membrane (MM).

2. MPD (-) and 7-AAD (-) - Live cells with depolarized MM.
3. MPD (+) and 7-AAD (-) - Live cells with intact MM.
4. MPD (-) and 7-AAD (+) -Dead cells with intact MM.

Enzymatic assays

Enzymes are the key players in every cellular function. Some enzymes are responsible for initiating the stress response, while others act as antioxidants to counteract the stress response. So, analyzing both sides yields a strong conclusion. In this context, the assessment of carbonyl, lipid peroxidation, nitrite, and reduced glutathione was carried out. Cells of standard count were seeded in the T25 flasks and incubated for the 3/4th confluency. Glucose 50 mM was added to the flask and incubated for 24 hr. The untreated flask was kept as a control, and the ISN alone-treated group was maintained. The glucose-induced flask without treatment and the flask with treatment (ISN) were maintained. This treatment was followed for all the enzyme assays.

Carbonyl assay: Treated and untreated groups of cells subjected to carbonyl assay.²² where the cells were trypsinized to collect the cells, and the cell lysate was prepared using lysis buffer (Tris -0.1 M, EDTA -0.2 M, NaCl -2 M, and Triton X-100 -0.5 %). 500 μ L of cell lysate was mixed with 2,4-dinitrophenylhydrazine in 2M HCl and incubated for 60 min at RT with frequent vortexing every 10-15 min. 20% TCA of 500 μ L was added and centrifuged. The supernatant was discarded and the pellet was washed thrice using 1 mL of ethyl acetate with ethanol in a 1:1 ratio. The precipitated protein was dissolved in 600 μ L of Guanidine solution. Carbonyl content was calculated from maximum absorbance (390 nm) using the molar absorption coefficient of 22,000 M⁻¹ cm⁻¹.

Lipid peroxidation assay: LPO assay was performed as per the protocol suggested by Ohkawa *et al.*, 1979.²³ Cells from the treated and control groups were centrifuged and the pellet was mixed with 0.2 mL of lysis buffer as mentioned above and incubated for 15 -20 min at 4°C. 50 μ L of cell lysate mixed with 70% ethanol-0.5 mL and 1% TCA – 1 mL. The contents were then boiled for up to 20 min and cooled to room temperature using running tap water. Followed by 50 μ L of Acetone was added, and the absorbance was read at 535 nm.

NO assay: Nitrate assay was investigated by the slightly changed protocol of Lepoivre *et al.*, 1990.²⁴ Cell lysate was prepared as mentioned earlier. 0.5 mL of cell lysate was mixed with 3% sulphosalicylic acid and mixed for half an hour, followed by centrifugation at 5000 rpm for 15 min to neutralize the protein. 0.2 mL of supernatant was added to 10% NaOH -30 μ L and Tris HCl buffer-300 μ L. Griess reagent of 530 μ L (1% sulphanilamide, 2% phosphoric acid, and 0.1% N-1-naphthyl ethylene diaminedihydrochloride) was added to the mixture and incubated in the dark for 10-15 min, and then subjected to a microplate reader for a 540 nm absorbance reading against the

Griess blank. Sodium nitrite solution was utilized as the standard, and the concentration of nitrate liberation was measured using a standard curve.

GSH estimation: Cell lysate of treated and control groups was prepared as mentioned previously and the steps followed as prescribed by Moron *et al.*, 1979.²⁵ 1 mL of lysate was mixed with 0.5 mL of 0.2 M phosphate buffer of pH 8 and 0.2 mL of 0.6 mM DTNB. Top up with 1.3 mL of distilled water. The mixture was then read at 420 nm and compared with the standard graph performed with reduced glutathione.

Real-time PCR

Cells were seeded at a T25 cm² flask and incubated to reach appropriate confluency. The cells were exposed to 50 mM Glucose, then treated with ISN at a fixed concentration of 6.25 μ g/mL. ISN-treated and untreated cells were maintained. After the incubation period, the inducer, sample exposure, sample alone treated, and untreated groups proceeded with the RNA isolation procedure using TRIzol. Each flask was added with 1mL of TRIzol and kept for 5 min. To the cell pellet, 200 μ L of Chloroform was added and centrifuged. 0.5 mL of saturated isopropanol was added to the aqueous layer and left for 10 min at RT and centrifuged. The resultant pellet was washed with 70% ethanol twice by centrifugation at 4°C (Remi CM12). Tris-EDTA buffer was used to resuspend the final pellet, which was air-dried. Isolated RNA was analysed for its concentration and purity, then converted into cDNA by the iScript cDNA synthesis kit (Biosciences, Product code- 786-5019s, 786-5020, master premix for first-strand cDNA synthesis) using a Thermal cycler (Eppendorf Master Cycler). SYBR Green Master Mix (G Biosciences, Product code-786-5062) was used to perform qPCR analysis with the freshly synthesized cDNA. Light cycler 96 (Roche) was utilized to analyse gene expression. Master mix, cDNA template, forward and reverse primers of FOXO1 (primer sequence -F: 5'ACTTTTGATAATGTGTTGCC3', R: 5'CTGCTGTCAGACAATCTGA3') and GAPDH (primer sequence -F: 5'AATGCATCCTGCACCACCAACTGC3';R: 5'GGAGGCCATGTAGGCCATGAGGTC3') was added respectively and PCR conditions were set as initial activation (2 min) followed by denaturation at 60°C, Annealing (1 min) at appropriate primers at optimized temperature (Tm of FOXO1 -F: 53.2, R: 54.5 and GAPDH -F:64.4, R:67.8). Extension step (1 min) at 72°C for 34 cycles. All the experiments were triplicated. The $\Delta\Delta$ Ct method was used to calculate the gene expression.

Statistical analysis

The Result data output from all the experiments were triplicated and given as Mean $\pm$ SD. All the assay results were analyzed statistically by Graphpad PRISM. One-way ANOVA was performed and the group comparison was interpreted by Dunnett's multiple comparison test. The significance level was considered as $p < 0.05$.

RESULTS

Cytotoxicity assessment of ISN on HEK 293 cell line

Cytotoxicity assessment of ISN was performed by the MTT method. Cytotoxicity testing is the initial step in the study of a bioactive compound's effect against a particular cell line. Figure 1 depicts the graphical representation of the cytotoxicity potential of ISN towards the HEK 293 cell line for 24 hr. Untreated cells showed 100% viability, where ISN-treated cells exhibited a gradual decrease in viability in a dose-related manner. 6.25, 12.5, 25, and 50 $\mu\text{g/mL}$ showed 92.70 ± 0.820 , 85.26 ± 1.207 , 75.05 ± 0.856 , and 67.81 ± 0.978 % viability, respectively. The higher dosage 100 $\mu\text{g/mL}$ showed the 47.35 ± 1.254 percentage viability, which was scrutinized using ED_{50} PLUS V1.0 software to find the exact IC_{50} value. 93.50 $\mu\text{g/mL}$ exhibited the exact IC_{50} value. The result concludes that ISN has a cytotoxic effect against the HEK 293 cell line at higher concentrations, such as 93.50 $\mu\text{g/mL}$. Statistical analysis showed the treated groups were highly significant compared to the control group ($p < 0.01$). Figure 2 represents the morphological deterioration in a dose-dependent manner. Untreated cells showed fine morphology; treated cells exhibited cell shrinkage, detachment, elongation, and nuclear condensation at high doses such as 25, 50, and 100 $\mu\text{g/mL}$. Low dosage exposure showed very less or negligible changes in the morphology.

Nephroprotective effect of ISN

The nephroprotective potential of ISN was investigated using an MTT assay and is depicted in Figure 3. Graphical representation indicates that glucose-exposed cells showed a reduced viability percentage of 54.75 ± 4.37 compared to the control, showing 100 % viability. In contrast, the ISN drug showed an improved

viability percentage up to 6.25 $\mu\text{g/mL}$. High dosages like 12.5 and 25 exhibited a slight decrease in the cellular viability, as 81.17 ± 7.74 and 73.30 ± 5.60 , respectively. Low doses (1.5, 3.1, and 6.25 $\mu\text{g/mL}$) showed a gradual protective effect against glucose toxicity in HEK 293 cells, as indicated by increased viability (64.35 ± 5.30 , 71.48 ± 6.47 , and $84.94 \pm 9.15\%$, respectively). The result output confirms the nephroprotective effect of ISN undoubtedly. Statistical significance implies that 1.5 $\mu\text{g/mL}$ showed nonsignificance with the induced group, whereas 3.1 and 6.25 $\mu\text{g/mL}$ showed low significance and high significance with the induced group. High dosages of 12.5 and 25 $\mu\text{g/mL}$ showed high and low significance with the induced group, respectively. The induced group exhibited a high significance with the control group by Dunnett's multiple comparison test. (ns- non significant, * - $p < 0.05$, *** $p < 0.01$).

Dual staining -AO/EtBr assay

Figure 4 represents the AO/EtBr staining of the untreated, High glucose-induced, ISN-treated in HG-induced cells and ISN alone-treated groups. The control and ISN alone-treated group solely exhibit the complete live cells indicated by the most intense green fluorescence. HG group showed an elevated red fluorescence count, indicating necrotic and late apoptotic cells with elongated morphology and condensed/damaged nuclei, which was reduced by ISN treatment, as evidenced by the HG+ISN group, where the reduction in necrotic and late apoptotic cells was observed. The graphical denotation also showed a similar trend according to the intensity, where we can observe a high percentage of normal cells with a negligible amount of EA, LA, and Nec cells in the control and ISN alone groups. In contrast, the augmented percentage of cells was observed as LA and Nec in the HG group showed lower significance with the control group, # $p < 0.05$, and the effect was

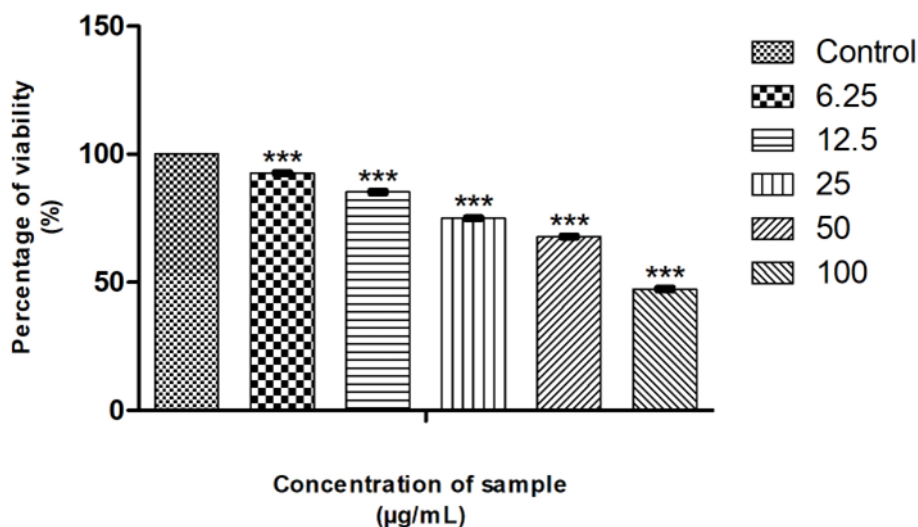


Figure 1: Cytotoxicity assessment of ISN on HEK 293 cell line. Graphical representation depicting the cytotoxicity effect of ISN on HEK293 cells by MTT assay. X axis - varied concentration of ISN and Y axis - % Viability. All experiments were done in triplicate and results are represented as Mean $\pm$ SD. One-way ANOVA and Dunnett's test were performed to analyse data. *** $p < 0.01$ compared to control groups.

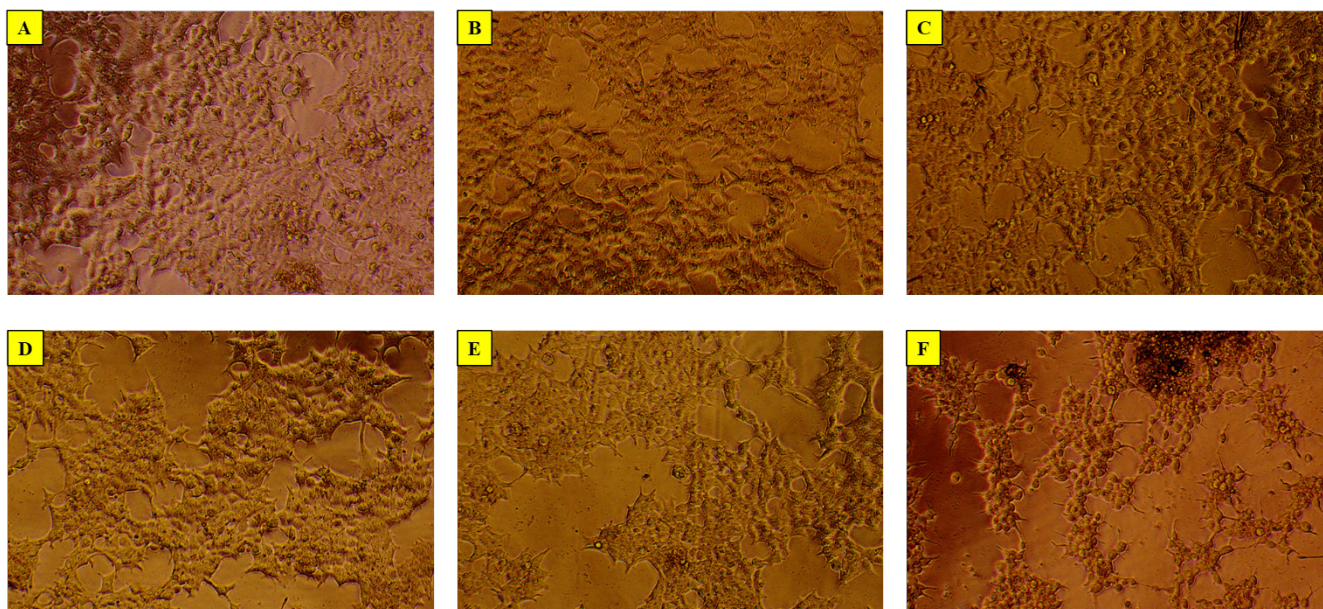


Figure 2: Morphological alteration treated with ISN. Morphological deterioration of HEK 293 cells upon treatment with ISN was shown in the Figure as A -control/untreated, B - F-6.25, 12.5, 25, 50 and 100 µg/mL, respectively.

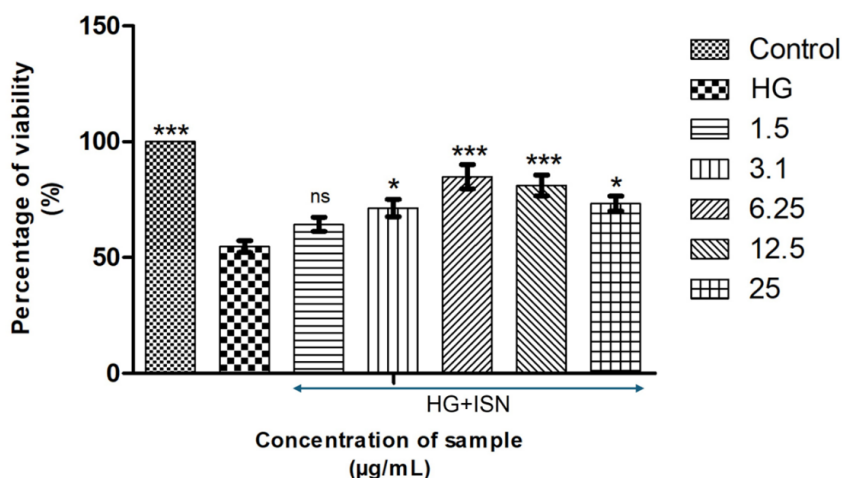


Figure 3: Nephroprotective effect of ISN on glucose-induced HEK 293 cell line. Graphical representation depicting the nephroprotective effect of ISN on the HEK293 cell line by MTT assay. X axis - varied concentration of ISN and Y axis - Percentage viability. All experiments were done in triplicate and results are represented as Mean±SD. One-way ANOVA and Dunn's test were performed to analyse data. *** $p < 0.01$ compared to high glucose induced groups, * $p < 0.05$ compared to high glucose induced groups, ns=non-significant compared to high glucose induced groups.

neutralized by the treatment of ISN exhibited in the HG+ISN group, with a low percentage of LA and Nec with the HG induced group * $p < 0.05$.

Comet Assay

A comet assay was performed to assess DNA damage in cells. Figure 5 showed the comet images of untreated, HG, HG + ISN, and ISN alone, respectively. Control and ISN groups showed the absence of a comet tail, which denotes that cells have intact DNA. HG-induced group exhibited the elevated comet length and tail length, as 118.772 ± 4.33 and 13.84 ± 2.98 µm, respectively, which denotes that the glucose exposure leads to the apparent

DNA damage. This adverse effect was neutralized by the treatment of ISN, which showed the reduced comet length and tail length as 70.223 ± 5.25 and 3.434 ± 0.62 µm, respectively. ISN has a great positive impact on the prevention of DNA damage from high glucose exposure. Dunnett's multiple comparison test demonstrated the low significance between the control-induced group (denoted as * $p < 0.05$) and the induced-treated group (denoted as # $p < 0.05$).

Mitochondrial membrane potential assay

Mitochondria are the major trigger point for initiating the intrinsic apoptosis signalling pathway. MMP Investigation is

essential in apoptosis research. The Mito potential kit is utilized to assess MMP. Figures 6A-6D depict the assessment results of MMP as control, HG group, HG + ISN group, and ISN alone group. From the results, it was very clear that the ISN treatment has a substantial protective impact on the mitochondrial membrane stability. Cell population percentage of untreated and ISN alone group exhibits a similar trend, showing a great percentage of live cells with almost 70%, fewer depolarized live cells in the range of 20-30 % and negligible depolarized dead/Dead cells percentage as 1% and 2.7 %. Induced group (HG) has a great adverse impact on the healthy cells, showing very few live cells, with a percentage of 22, which was recovered upon treatment with ISN, showing an improved percentage of live cells of 51 %. Similarly, ISN treatment also reduced the adverse effect caused by HG, reflected in the declining percentage of depolarized live/dead and dead, as shown in Figures 6B and 6C, respectively. Thus, ISN prevents cells from entering the apoptotic cascade.

Enzymatic assays

Enzymes play a pivotal role in most of the cellular mechanisms. Changes in the enzyme release have a great impact on the regular cellular activity. Thus, the assessment of the level or release of the enzyme was a predominant one. Oxidative equilibrium mainly depends on the balancing of oxidative stress enzymes and antioxidant enzymes. Carbonyl content was estimated and the result is shown in Figure 7. Augmented carbonyl release was observed in the HG group as 75.04 ± 2.11 nmol/mg protein, which was alleviated by the ISN treatment as 53.37 ± 3.97 nmol/mg protein. The control and ISN alone-treated group showed moderate levels, such as 19.14 ± 1.08 nmol/mg protein and 31.60 ± 1.96 nmol/mg protein, respectively. Similarly, in the LPO

assay, the HG group exhibited a high level of MDA units, as 2.975 ± 0.8 MDA nmol/mg protein, which was reduced with the treatment of ISN as 1.859 ± 0.74 MDA nmol/mg protein. Control and ISN showed very low levels of 0.871 ± 0.37 MDA nmol/mg protein and 0.990 ± 0.08 MDA nmol/mg protein, respectively. NO result output also followed the same trend as carbonyl and LPO assays, where high NO release of HG (579.15 ± 60.14 $\mu\text{g/mL}$) was reduced after treating with ISN (485.1 ± 24.63 $\mu\text{g/mL}$). The control and ISN group showed 242.55 ± 16.07 $\mu\text{g/mL}$ and 277.2 ± 16.99 $\mu\text{g/mL}$, respectively. In contrast, reduced glutathione exhibited an increased level of release at the control and ISN alone treated groups, as 7.234 ± 0.94 GSH $\mu\text{M/mg}$ protein and 6.050 ± 0.54 GSH $\mu\text{M/mg}$ protein, respectively. Glutathione was greatly reduced in the high glucose-induced group, with 3.147 ± 0.24 GSH $\mu\text{M/mg}$ protein, which was recovered by ISN treatment, and it was escalated to 5.124 ± 0.3 GSH $\mu\text{M/mg}$ protein.

Statistical significance was performed for all the assays by one-way ANOVA, where all the enzyme analyses showed a similar pattern, as the high glucose induced group showed low significance with the control group, denoted as * $p < 0.05$ and ISN treatment in the HG induced group (HG + ISN) showed low significance with the HG group, denoted as # $p < 0.05$.

Real-time PCR

Molecular-level analyses serve as a critical component, providing strong supportive evidence for the gene-level findings. FOXO1 is an important gene that plays the main role in glucose and lipid metabolism. It is also involved in many other cellular mechanisms. The FOXO1 gene was examined for control, HG, HG + ISN and ISN alone groups depicted in Figure 8. Graphical representation

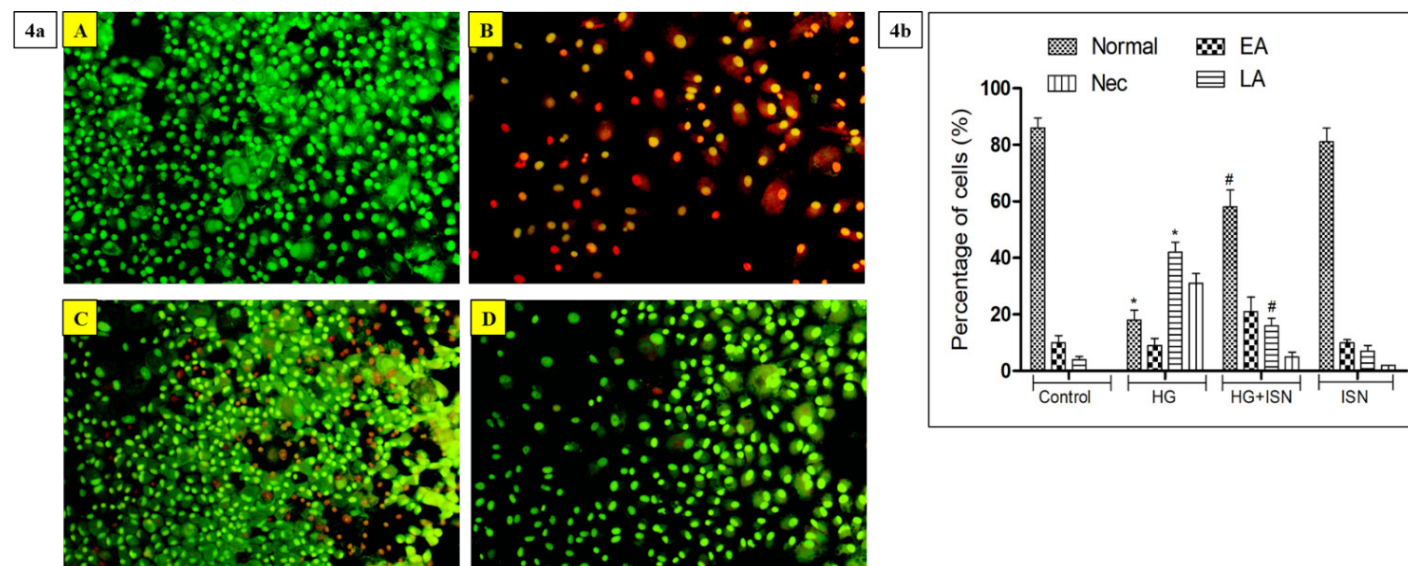


Figure 4: AO/EtBr staining of HEK 293 cells. Figure a represents AO/EtBr dual staining was performed for the various treatment groups as A -Untreated/control group, B -HG group, C - HG + ISN group and D -ISN group. Figure b shows a graphical representation of the quantitative result of the cellular population with statistical analysis. All experiments were done in triplicate and results are represented as Mean $\pm$ SD. One-way ANOVA and Dunnett's test were performed to analyse data. * $p < 0.05$ compared to control group, # $p < 0.05$ compared to high glucose induced groups. EA -early apoptotic cells, Nec -Necrotic cells and LA -Late apoptotic cells.

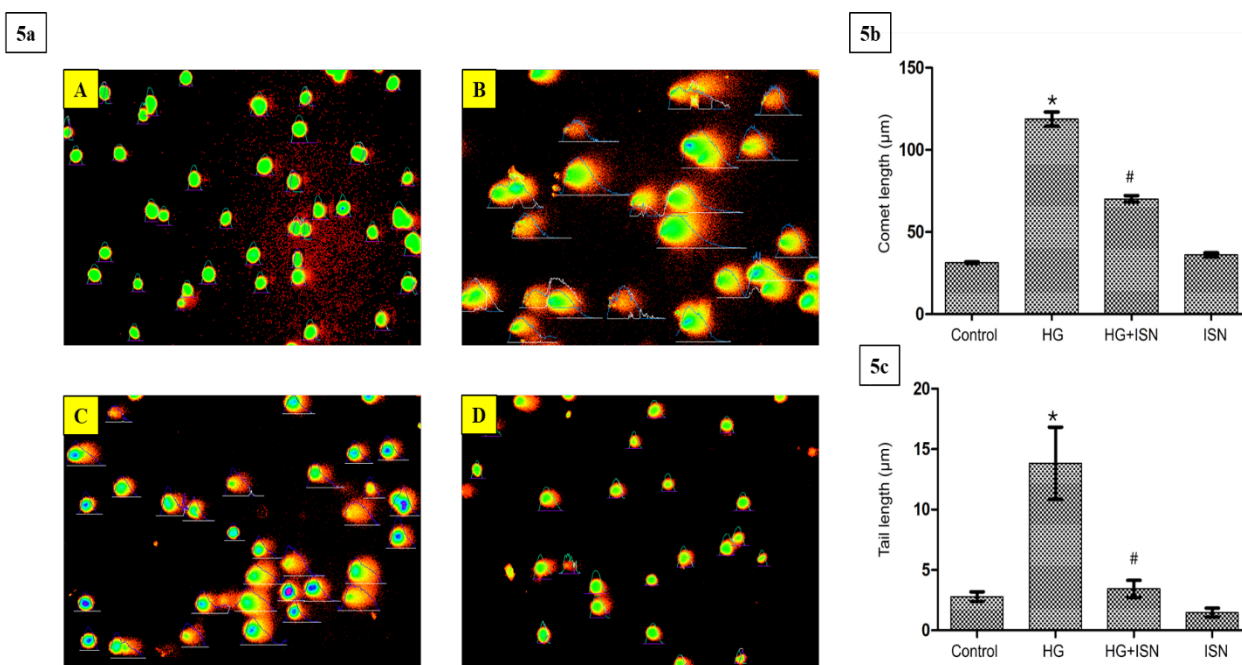


Figure 5: Comet assay. a: depicts the comet images produced by A - control/untreated group, B -HG, C -HG + ISN, and D -ISN, respectively. B and C: showed the graphical representation of comet head length and comet tail length, respectively, upon treatment with ISN. All experiments were done in triplicate, and results are represented as Mean±SD. One-way ANOVA and Dunnett's test were performed to analyse data. * $p < 0.05$ compared to control group, # $p < 0.05$ compared to high glucose induced groups.

clearly indicates the great reduction in the gene expression level at the high glucose-exposed group, which was levelled up by the treatment of ISN as 1.4-fold. Control and ISN alone-treated groups showed upregulated FOXO1 gene expression compared with the reference/housekeeping gene of GAPDH.

DISCUSSION

The current study demonstrates that Isorhamnetin (ISN) effectively attenuates high-glucose-induced diabetic nephropathy-associated cellular damage, as evidenced by multiple experimental parameters. The growing burden of diabetic nephropathy highlights the urgent need to identify novel and efficacious therapeutic agents capable of attenuating disease progression. Globally, the incidence of newly diagnosed patients requiring dialysis, renal surgical interventions, and kidney transplantation continues to rise, placing substantial strain on healthcare systems. Despite advances in clinical management, the lack of specific and targeted pharmacological therapies for diabetic nephropathy remains a major limitation.²⁶ Therefore, the development of safe, cost-effective, and mechanistically targeted therapeutic agents is of paramount one.²⁷ Diabetic nephropathy progresses through distinct stages of renal impairment, and early diagnosis coupled with timely intervention can significantly improve clinical outcomes.²⁸ In contrast, delayed detection often results in irreversible renal damage and progression to advanced disease stages. Diabetic Kidney Disease (DKD) is caused mainly by high glucose accumulation. To mimic the process, in this

study, a high glucose concentration of 50 mM was used to induce a hyperglycaemic model, which was supported by previous research studies.

ISN was assessed for cytotoxicity in HEK 293 cells, with an Inhibitory Concentration 50 (IC₅₀) near 100 μg/mL at higher dosages. Hence, for the nephroprotective assessment, a low non-toxic dosage of ISN was chosen as 6.25 μg/mL. Cytotoxicity assays confirm the nephroprotective properties of ISN. Morphological deteriorations and cell shrinkages in the HG-induced group were recovered to normal by the treatment of ISN. Apoptosis investigation done by dual staining demonstrated the heightened nuclear condensation in the HG group, which was significantly attenuated with the help of ISN. Chen *et al.* (2025) reported that ISN has the capacity to reverse the EMT process in both *in vivo* and *in vitro*, as rodent and HK2 cells, respectively, by inhibiting the TWEAK/Fn14 pathway (*in vivo*) and TGF β1, E-cadherin downregulation.²⁶

Comet assay showed the elongated comet structure with increased head and tail length in the HG group. The ISN-treated group exhibited reduced head and tail length, indicating that ISN repaired DNA damage. Hence, ISN proved once again in the protective effect on genotoxicity by involvement in DNA repair kinetics. A similar result was obtained for Lycopene in mitigating the high-glucose effect in NRK-52E kidney cells. High concentration of glucose always releases an enormous amount of Advanced Glycation End products (AGE), leading to damage to the DNA molecule.^{5,28,29}

Mitochondrial Membrane Potential (MMP) is a critical indicator of mitochondrial integrity and cellular survival under stress conditions. A higher concentration of glucose impairs haemostatic equilibrium, leading to the release of large amounts of free radicals. It disrupts intracellular redox homeostasis, leading to excessive generation of Reactive Oxygen Species (ROS) and subsequent mitochondrial dysfunction. It eventually creates pores in the Mitochondrial Membrane (MM), leading to the loss

of potential of the mitochondrial membrane.³³ Prolonged and sustained free radicals trigger the process of apoptosis, necrosis, and autophagy.²⁹ ROS and lipid radicals lead to the generation of MM pores, which in turn activate the Bcl₂ gene, directing the initiation of the caspase cascade pathway. In the present study, high glucose exposure markedly increased the population of depolarized and dead cells, indicating mitochondrial destabilization in HEK293 cells. Treatment with isorhamnetin

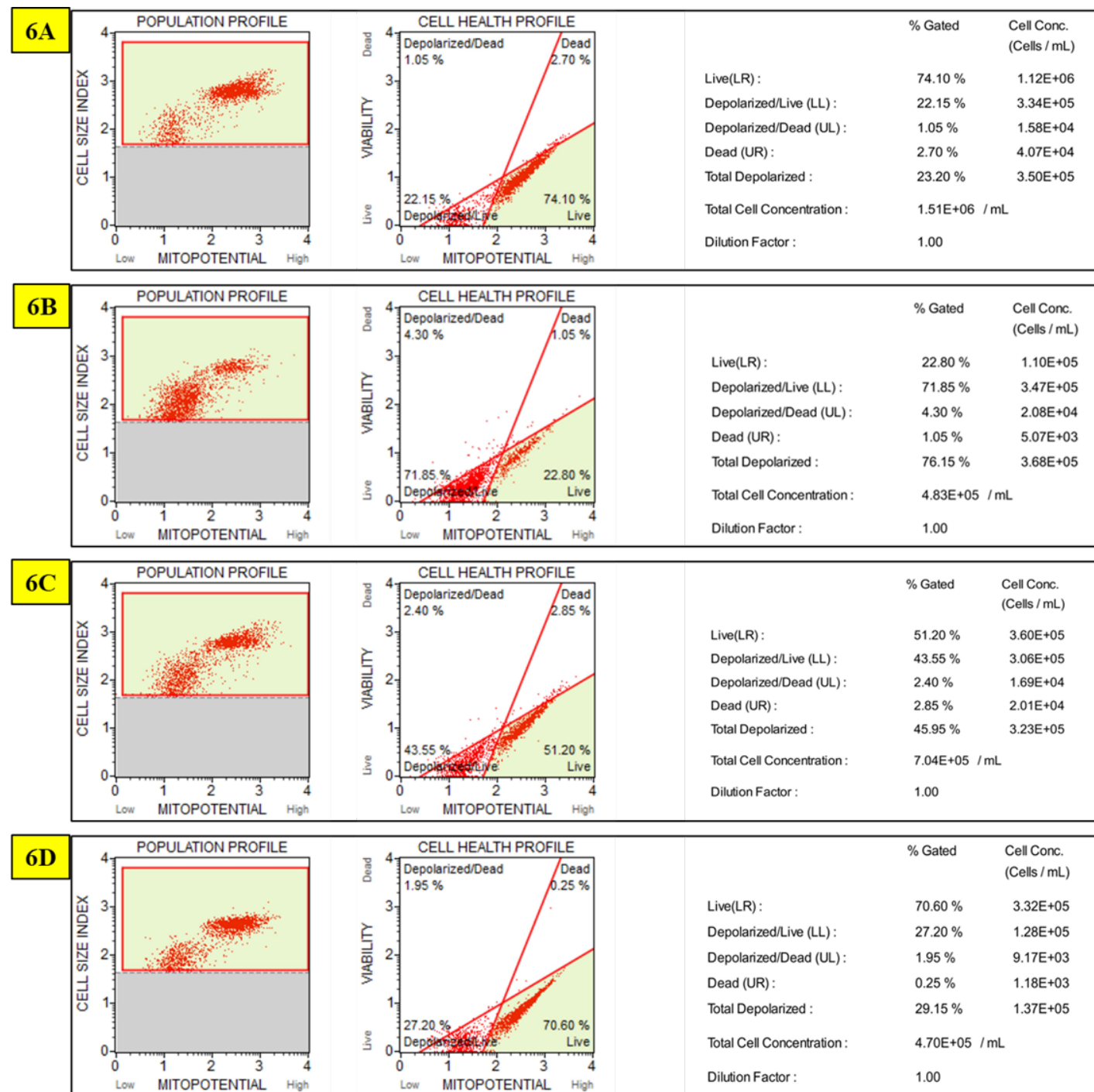


Figure 6: MMP assessment. 6A) Control, 6B) HG, 6C) HG + ISN and 6D) ISN alone group. MMP results showed that ISN treatment has a significant positive impact on the recovery of cells from the apoptotic cycle. Images showed population and health profiles, with data on live, depolarized live, depolarized dead, and dead cell percentages.

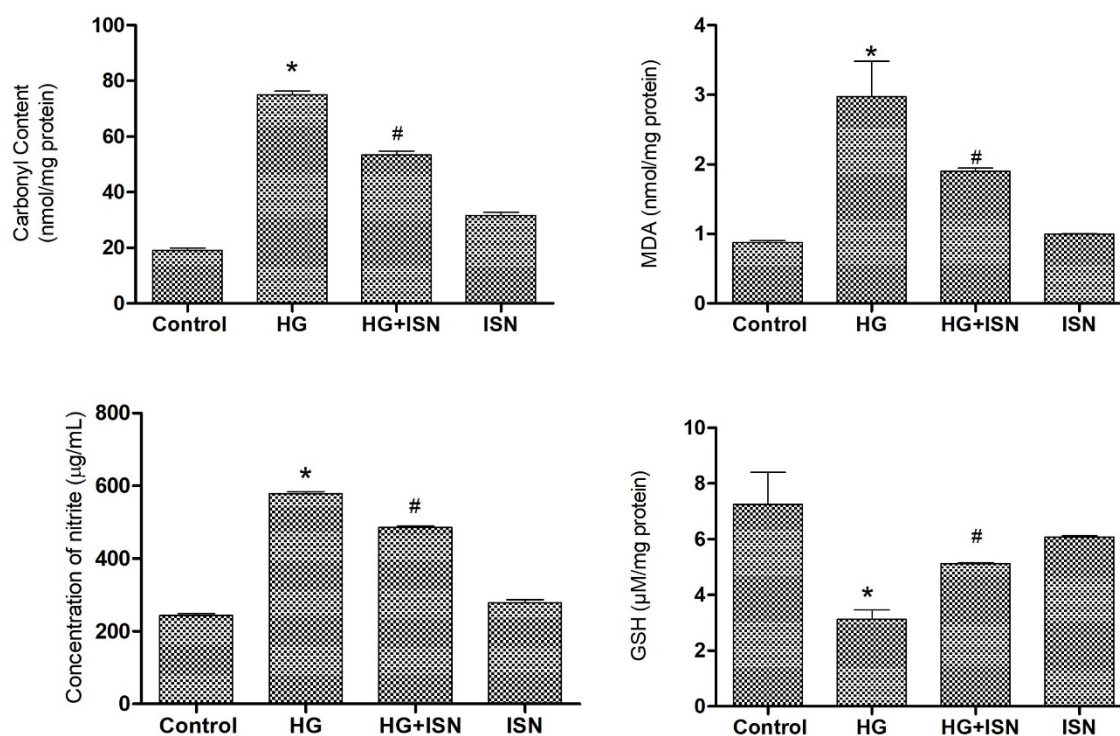


Figure 7: Enzymatic investigation. Graphical representation depicting the Protein carbonylation, Lipid peroxidation, Cellular Nitrite and GSH levels of untreated control, high glucose exposed, high glucose exposed with ISN treatment and ISN treatment alone groups. All experiments were done in triplicate and results are represented as Mean $\pm$ SD. One-way ANOVA and Dunn's test were performed to analyse data, where * indicates the comparative statistical analysis between the control and induced group. # indicates the comparison between the HG + ISN group and the HG induced group.

significantly restored MMP, as evidenced by an increased proportion of cells with intact mitochondrial membranes and a concomitant reduction in depolarized and dead cell populations. These findings suggest that isorhamnetin preserves mitochondrial integrity under hyperglycaemic conditions, thereby limiting mitochondrial-mediated apoptotic progression. Similar observations have been reported by Eslami and Sharifi (2012), who investigated the impact of high glucose 100 mM on HEK 293 cells. They noted that HEK 293 cells were strongly affected by high glucose concentrations, leading to activation of the Bax protein via opening of mPTP (mitochondrial Permeability Transition Pores), followed by cytochrome c release, which is guided to the caspase 3-dependent pathway.³¹

As we discussed earlier, a higher concentration of glucose uptake led to an enormous release of free radicals, which disrupted the whole cellular functional system. Free radicals give an unnecessary hit to each and every cellular process, leading to the perplexity of cellular function. These imbalances tend to reduce anti-oxidant enzymes greatly GSH. In contrast, oxidative stress enzymes like carbonyl, Nitrite and lipid peroxidase were increased tremendously. In our study, the HG group showed an upregulation of oxidative stress enzymes, whereas antioxidant enzymes showed the opposite trend. ISN exposure strongly supports reducing stress enzymes such as Lipid peroxidation, carbonyl, and Nitrite, and conversely increases the availability of GSH to balance the

equilibrium. QIU *et al.*, 2015, Jamali-Raeufy *et al.*, 2019, Kalai *et al.*, 2023, Esam Qnais *et al.*, 2024 and Alqudah *et al.*, 2025 reported that ISN greatly reduced the lipid peroxidation release and elevated the total SOD, CAT and GSH in diabetic rat denotes ISN has the remarkable anti-oxidant property. Also, ISN inhibits the NF- κ B signalling pathway, thereby mitigating the effects of inflammation while preventing renal impairment.^{10,14,15,17,32} Many other flavonoids, such as quercetin, apigenin, baicalin, luteolin, diosmin, and kaempferol, also follow a similar trend, as ISN protects renal cells by mitigating the hyperglycaemic impact and oxidative stress in DN.⁶

FOXO1 is a key transcription factor involved in the regulation of glucose metabolism, oxidative stress response, and cellular homeostasis in renal cells. Under hyperglycaemic conditions, suppression of FOXO1 expression has been associated with increased oxidative damage, mitochondrial dysfunction, and enhanced susceptibility to apoptosis. In the present study, high glucose exposure significantly downregulated FOXO1 mRNA expression in HEK293 cells, indicating impaired transcriptional regulation under diabetic stress conditions. Treatment with ISN evidently restored FOXO1 gene expression. This suggests a protective transcriptional modulation in response to hyperglycemia. This restoration of FOXO1 expression correlated with reduced oxidative stress markers, improved mitochondrial membrane potential, and decreased apoptotic features,

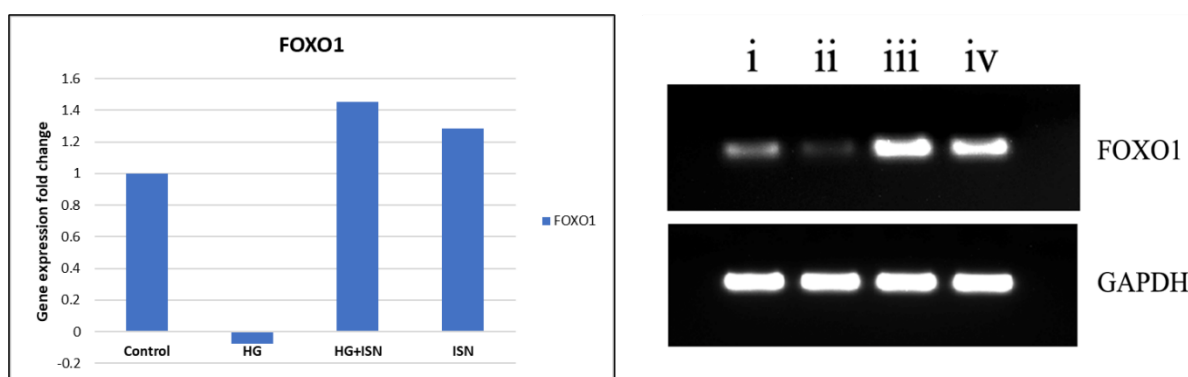


Figure 8: Gene expression analysis of FOXO1. Graphical representation depicting the expression pattern of FOXO1 in different.

supporting the idea that FOXO1-associated mechanisms may contribute to the nephroprotective effects of isorhamnetin. Previous studies have reported that FOXO1 plays a crucial role in attenuating epithelial-mesenchymal transition, fibrosis, and apoptosis in diabetic kidney disease models, further supporting its relevance in renal protection.^{18,19,33} However, as FOXO1 expression was evaluated only at the mRNA level in this study, the present findings indicate an association rather than direct causal evidence. Further investigations involving protein expression, post-translational modifications, downstream target analysis, and loss-/gain-of-function approaches are warranted to confirm the precise mechanistic role of FOXO1 in isorhamnetin-mediated renoprotection. Collectively, Isorhamnetin effectively protects HEK 293 cells from high-glucose-induced injury, suggesting its potential as a promising nephroprotective candidate for diabetic nephropathy. However, the present findings are limited to an *in vitro* study using HEK-293 cells, which may not fully recapitulate the physiological characteristics of mature renal tubular cells. Therefore, further investigations using more relevant renal cell models and *in vivo* studies are required to evaluate pharmacokinetic behaviour, bioavailability, tissue distribution, dose optimization, and therapeutic efficacy before clinical translation.

CONCLUSION

In conclusion, isorhamnetin attenuated high-glucose-induced oxidative stress and apoptosis in HEK293 cells, potentially through modulation of FOXO1-associated signalling pathways. While these findings highlight its nephroprotective promise, the study is limited to an *in vitro* model; therefore, further *in vivo* validation, pharmacokinetic evaluation and detailed pathway-specific investigations are required to confirm its therapeutic relevance in diabetic nephropathy.

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ABBREVIATIONS

ACE: Angiotensin-Converting Enzyme; **AGE:** Advanced Glycation End Products; **AO:** Acridine Orange; **ARBs:** Angiotensin Receptor Blockers; **CKD:** Chronic Kidney Disease; **cDNA:** Complementary DNA; **DM:** Diabetes Mellitus; **DMEM:** Dulbecco's Modified Eagle's Medium; **DN:** Diabetic Nephropathy; **DKD:** Diabetic Kidney Disease; **DMSO:** Dimethyl Sulfoxide; **DTNB:** 5,5'-Dithiobis-(2-nitrobenzoic acid); **EA:** Early Apoptotic Cells; **ED₅₀:** Effective Dose 50; **EMT:** Epithelial-Mesenchymal Transition; **ER:** Endoplasmic Reticulum; **ESRD:** End-Stage Renal Disease; **EtBr:** Ethidium Bromide; **FACS:** Fluorescence-Activated Cell Sorting; **FBS:** Fetal Bovine Serum; **FOXO1:** Forkhead Box O1; **Fn14:** Fibroblast Growth Factor-Inducible 14; **GAPDH:** Glyceraldehyde-3-Phosphate Dehydrogenase; **GSH:** Reduced Glutathione; **HG:** High Glucose; **HK-2:** Human Kidney Proximal Tubular Cell Line; **HEK293:** Human Embryonic Kidney 293 Cells; **H₂S:** Hydrogen Sulfide; **IC₅₀:** Inhibitory Concentration 50; **IDF:** International Diabetes Federation; **ISN:** Isorhamnetin; **LA:** Late Apoptotic Cells; **LPO:** Lipid Peroxidation; **MDA:** Malondialdehyde; **MMP:** Mitochondrial Membrane Potential; **MPD:** Mito Potential Dye; **mPTP:** Mitochondrial Permeability Transition Pore; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **NaOH:** Sodium Hydroxide; **NFKB:** Nuclear Factor Kappa B; **NMA:** Normal Melting Agarose; **NO:** Nitric Oxide; **NRK-52E:** Normal Rat Kidney Epithelial Cell Line; **PBS:** Phosphate-Buffered Saline; **PCR:** Polymerase Chain Reaction; **qRT-PCR:** Quantitative Real-Time Polymerase Chain Reaction; **RIF:** Renal Interstitial Fibrosis; **RNA:** Ribonucleic Acid; **ROS:** Reactive Oxygen Species; **RT:** Room Temperature; **SA:** Sodium Alginate; **SD:** Standard Deviation; **SE:** Standard Error; **SGLT2:** Sodium-Glucose Cotransporter-2; **STZ:** Streptozotocin; **T25:** 25 cm² Tissue Culture Flask; **TCA:** Trichloroacetic Acid; **TGF-β:** Transforming Growth Factor Beta; **Tris:** Tris(hydroxymethyl)aminomethane; **WHO:** World Health Organization; **7-AAD:** 7-Aminoactinomycin D.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

Lin Zhang, Meilan Zhou, Amirah Albaqami, Omar Oraibi, Mohammed Somaili, May Mireani: Conceptualization, Methodology, Investigation, Writing -Original Draft, Supervision, Review and Editing. All authors read and approved the final manuscript.

SUMMARY

This study investigated the nephroprotective potential of the flavonoid isorhamnetin against high-glucose-induced injury in Human Embryonic Kidney (HEK293) cells. Hyper glycaemic exposure induced cytotoxicity, oxidative stress, mitochondrial dysfunction, DNA damage, and apoptosis, accompanied by downregulation of FOXO1 expression. Low-dose isorhamnetin significantly restored cell viability, preserved mitochondrial membrane potential, reduced oxidative stress markers, enhanced antioxidant defenses, and attenuated apoptotic and genotoxic damage. Importantly, isorhamnetin reversed FOXO1 suppression under hyper glycaemic conditions, suggesting a mechanistic role in cellular protection. These findings demonstrate that isorhamnetin attenuates diabetic nephropathy-like injury *in vitro*, supporting its potential regulatory role of FOXO1-modulating nephroprotective agent.

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