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Isopulegol mitigates high glucose-induced oxidative stress in HK-2 cells via activation of the Nrf2/ARE pathway

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ABSTRACT

Diabetic nephropathy (DN) represents a major contributor to chronic kidney disease, primarily resulting from oxidative stress and injury to renal tubules triggered by persistent hyperglycemia. This study evaluated the protective effects of isopulegol (IPG), a monoterpene on high glucose (HG)-induced cytotoxicity in HK-2 cell lines. Cytotoxicity assays (MTT and CCK-8) revealed the effect of IPG on cell viability in HK-2 cells under HG conditions, with an IC_{50} of 45 µM. Treatment with IPG significantly improved the enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)] and glutathione (GSH) level, while reducing lipid peroxidation (LPO). Additionally, IPG promoted expression of Nrf2, activating the antioxidant response element (ARE) pathway. These findings suggest that IPG confers cytoprotective effects by enhancing antioxidant defense, reducing oxidative stress, highlighting its therapeutic potential in preventing oxidative renal damage in diabetic nephropathy.

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INTRODUCTION

Diabetes mellitus (DM) is an endocrine and metabolic disorder that poses significant risk to human health. As reported by the International Diabetes Federation (IDF), the present prevalence of diabetes is at 537 million individuals, with a projected rise of 784 million by 2045 (Roy *et al.*, 2024). Complications of diabetes mellitus include retinopathy, cardiovascular disease, and nephropathy (Alicic *et al.*, 2017).

Globally, diabetic nephropathy (DN) is emerging as th e primary factor causing end stage renal disease (ESRD) and a significant contributor to illness and death in diabetic individuals.

DN has a complex etiology characterized by alterations in renal hemodynamics, malfunction of the polyol system, oxidative stress (OST) and stimulation of inflammatory cascades. Mitochondrial impairment and the progression of OST are widely regarded as the primary pathogenetic mechanisms that trigger and advance DN (Chen *et al.*, 2017). OST arises when the equilibrium between pro-oxidants and antioxidants shifts towards the dominance of pro-oxidants manifested as an abundance of reactive oxygen species (ROS).

An excessive amount of ROS can harm cellular structures, therefore leading to the development of diseases (Finkel, 2011). Sustained hyperglycemia through ROS contributes to OST via weakening the cellular antioxidant defense mechanisms. This imbalance causes oxidative injury to biomolecules and activates immune responses, promoting the release of inflammatory mediators and cytokines. These inflammatory processes adversely affect glomerular capillaries and disrupt renal tubular architecture and function, thereby accelerating the decline in overall renal performance (Jin *et al.*, 2023).

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is crucial for maintaining redox balance through its regulation on antioxidant and detoxification pathways that combat oxidative stress. Previous studies have emphasized the crucial involvement of the Nrf2/antioxidant response element (Nrf2/ARE) signaling pathway in modulating cellular antioxidant defense systems. Findings from Nrf2 knockout mouse models have demonstrated that loss of Nrf2 function heightens susceptibility to oxidative stress–related disorders, notably contributing to the development of conditions such as colorectal cancer, DN and chronic obstructive pulmonary disease. In its physiological state, Nrf2 in association with Keap1, the repressor protein remains dormant in the cytoplasm (Tan and de Haan, 2014). OST promotes the translocation of Nrf2 to the nucleus activating AREs, driving the expression of genes involved in cellular defense, such as HO-1 and SOD (Kim and Vaziri, 2010). Previous findings collectively suggest Nrf2 as a promising therapeutic target for the prevention and management of DN (Ma, 2013).

Conventional pharmacological treatments for DN are often associated with adverse side effects. The rapidly increasing global incidence of DN has intensified the need to explore complementary and alternative therapeutic strategies. In this context, phytochemicals have gained significant attention as promising natural agents with broad therapeutic potential, particularly in mitigating DN (Li *et al.*, 2021). Monoterpenes are naturally occurring compounds found in herbs, vegetables and fruits that exhibit antioxidant, anti-inflammatory and analgesic activities (Sousa *et al.*, 2023). Recent studies highlight monoterpenes as promising therapeutic agents capable of modulating enzymes and proteins involved in insulin resistance and the pathological mechanisms underlying diabetic nephropathy (Al Kury *et al.*, 2022). Isopulegol is a monoterpene found in the essential oils derived from *Citrus acidavar, Citrus paradisi* and *Corymbia citriodora* (Ou *et al.*, 2015; Salem *et al.*, 2018). The structure of IPG, a member of p-menthane family indicates the existence of two conjugated isoprene units and one oxygen atom at the third position. The pharmacological activities include antioxidant, antifungal, antibacterial, antispasmodic, antidiabetic and anticancer effects (Kalaivani and Sankaranarayanan, 2019; Fonsêca

et al., 2020). In this context, the study was designed to investigate the effect of IPG on OST markers in HG-exposed HK-2 cell lines.

MATERIALS AND METHODS

Maintenance of cells

HK-2 cells were cultured in a medium supplemented with 10% FBS, 1% penicillin-streptomycin solution and incubated at 37°C with 5% CO_2 to maintain appropriate temperature and gas levels for optimal cell growth. Cells were grouped as follows: Normal control group (NG group): 5.6 mmol/L glucose; Osmotic pressure control group (OP group): 5.6 mmol/L glucose + 24.4 mmol/L mannitol; High glucose group (HG group): 30 mmol/L glucose; High glucose group + different concentrations of IPG (10,20,30 µM) (HG + IPG group). After 48 h of HG and IPG treatment, the cell lysates were collected for subsequent experiments (Ma *et al.*, 2022).

Measurement of cell viability

MTT assay

Briefly, 5 × 10^3 HK-2 cells were cultured in a 96-well plate for 48 h and treated with medium containing graded concentrations of isopulegol (10 –100 µM). Following 48 h incubation under standard conditions (37 °C, 5% CO_2) MTT was added and the formazan formed were dissolved in DMSO after 3 h incubation. Percentage of viability were calculated (Mosmann, 1983).

CCK -8 assay

In CCK-8 assay, HK-2 cells maintained as per experimental design were incubated with 10 µL of CCK-8 reagent (4 h, 37°C) and the absorbance was recorded at 450 nm (Fan *et al.*, 2024).

Assessment of enzymatic and non-enzymatic antioxidants

The activities of SOD, CAT, GPx, GST and the level of GSH were quantified in the HK-2 cells treated with IPG (30µM) using commercial assay kits according to the manufacturers' protocols (Kakkar *et al.*, 1984; Sinha, 1972; Rotruck *et al.*, 1973; Habig *et al.*, 1974; Ellman, 1959).

Lipid peroxidation assay

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) levels using a commercial assay kit, following the manufacturer's instructions (Niehaus and Samuelsson. 1968). HK-2 cells plated in 6-well plates at a density of 8 × 10^4 cells per well were treated with isopulegol (30µM) and high glucose (HG) according to the cell viability assay protocol. Post-treatment, cells were washed thrice with PBS and homogenized in 0.5 mL of buffer. The homogenates were centrifuged for 10 minutes at 4000 × g and supernatants collected for MDA determination. For MDA measurement, 600 µL of thiobarbituric acid (TBA) solution was added to each sample tube to form MDA-TBA complexes, incubated at 95°C for 60 minutes, cooled on ice for 10 minutes and absorbance recorded at 532 nm using a microplate reader.

Detection of ROS

The intracellular ROS in HK-2 cells was measured using the dye DCFH-DA. The experimental cells were treated with dye and incubated for 30 minutes at 37°C. Fluorescence intensity was measured at 485 ± 10 nm (excitation wavelength) and 530 ± 12.5 nm (emission wavelength) using a microplate reader and ROS generation was visualized under a fluorescence microscope equipped with a blue filter (450–490 nm) (Suliman *et al.*, 2015).

Western blotting

The cells from various experimental groups were lysed with RIPA buffer and the protein content was quantified using Bradford's method (Kruger, 2009). Proteins were electrophoresed by 5% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Hercules, CA). Membranes were blocked with 5% non-fat milk prepared in PBS containing 0.05% Tween-20 for 1 h at room temperature and incubated overnight with the primary antibody for Nrf2 (1:1000 dilution, Santa Cruz Biotechnology, Inc.) at 4°C. Membranes were treated with secondary antibody (HRP-conjugated;1:5000 dilution) after washing with PBS-T. Protein bands were visualised using ECL kit

(Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol and the band intensities were measured with Image J software.

Statistical analysis

Assays carried out in triplicates were expressed as mean ± standard deviation (SD) and the difference between the groups were evaluated by one-way ANOVA followed by Tukey's post hoc test (p-value < 0.05). Data were analysed using GraphPad Prism software version 8.0 (GraphPad Software Inc., CA, USA).

RESULTS

Effect of IPG on HK-2 cell viability

IPG exhibited a dose dependent effect (10-100 µM) on the viability of HK-2 cell. The IC_{50} value was found to be 45µM (Fig. 1).

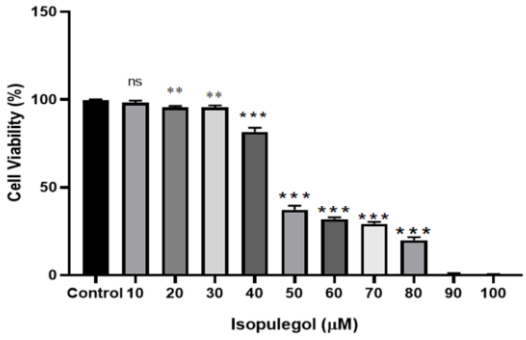


Fig. 1. Role of IPG on viability of HK-2 cells using MTT assay. Values are expressed as mean ± SD (n = 3) and the difference between the groups were evaluated by one – way ANOVA followed by Tukey's Post-hoc test. ns – no significance, ** $p < 0.05$ vs control, *** $p < 0.05$ vs control.

CCK-8 assay

A dose-dependent reduction in viability of HK-2 cells with IPG was observed after 48 h, with an IC_{50} value of 45µM. HG group (30 mM glucose) showed significantly reduced viability compared to control (5.6 mM glucose). IPG treatment (10, 20 and 30µM) did not affect viability under normal glucose condition but significantly restored cell viability in HG-treated cells in a concentration-dependent manner. The findings revealed that 30µM of IPG exhibited a significant effect than other two doses. Based on this, 30µM of IPG was utilized for further studies (Fig. 2).

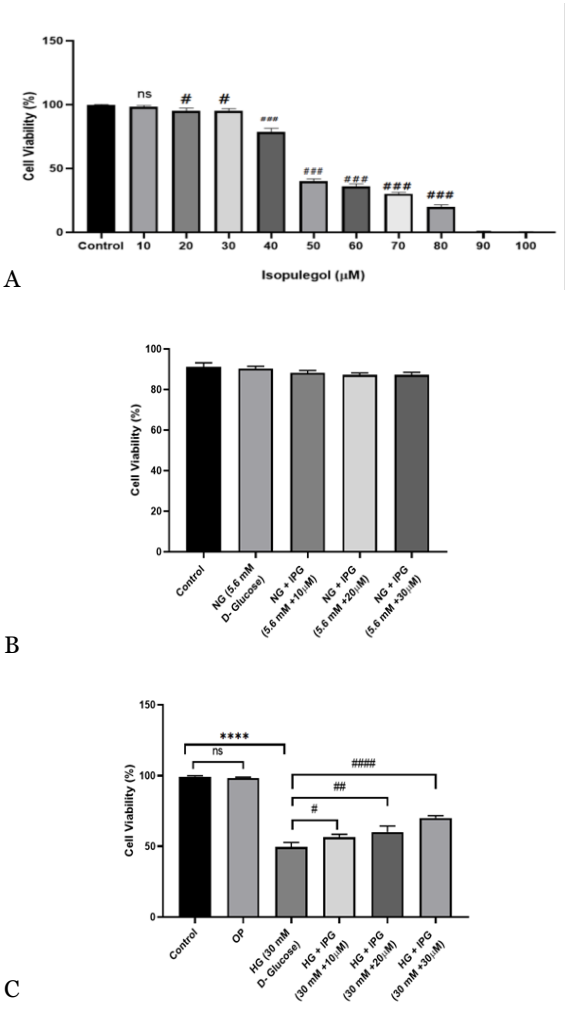
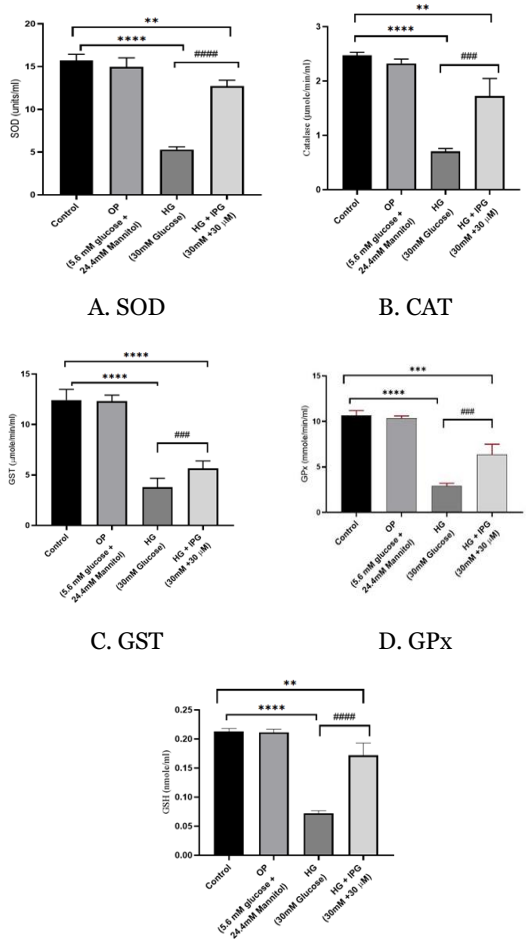


Fig. 2. Cell viability assessment by CCK-8 assay in HK-2 cells. A. Different concentrations (10 - 100µM) of IPG were treated with HK-2 cells**,** B. Normal Glucose (5.6mM) and a series of IPG (10,20,30µM), **C**. High Glucose (30mM) and a series of IPG (10,20,30µM). Values are expressed as mean ± SD (n = 3) and the difference between the groups were evaluated by one – way ANOVA followed by Tukey's Post-hoc test. A) ns – no significance, # $p < 0.05$, ### $p < 0.05$ vs control, C) ns – no significance, **** $p < 0.05$ vs control, # $p < 0.05$, ## $p < 0.05$, #### $p < 0.05$ vs HG.

Effect of IPG on oxidative stress markers in experimental cell lines

In HG-exposed HK-2 cells the enzymatic (SOD, CAT, GST, GPx) and non-enzymatic antioxidants (GSH) were

significantly decreased when compared to normal control. On exposure with IPG (30µM), the antioxidant levels were significantly improved (Fig. 3).



A. SOD B. CAT

C. GST D. GPx

E. GSH

Fig. 3. Effect of IPG on antioxidant system in HK-2 cell lines. Values are expressed as mean ± SD (n=3) and the difference between the groups were evaluated by one – way ANOVA followed by Tukey's Post-hoc test. $^{**}p < 0.05$, $^{***}p < 0.05$, $^{****}p < 0.05$ vs control, ### $p < 0.05$, #### $p < 0.05$ vs HG.

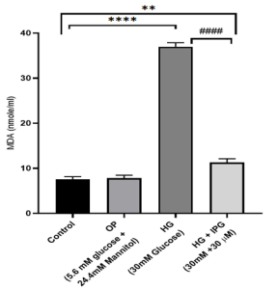


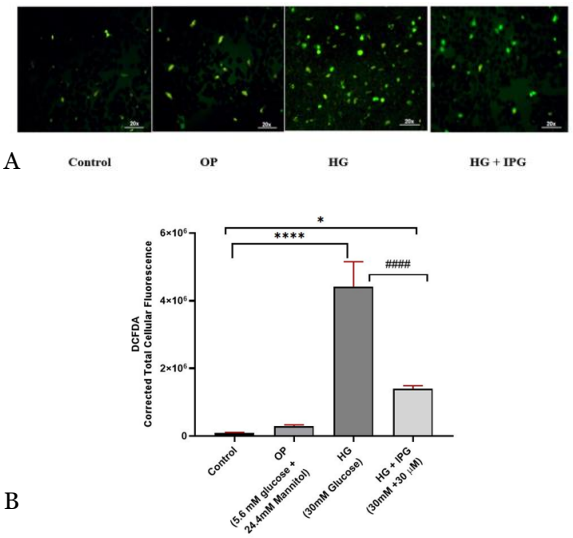
Fig. 4. Effect of IPG on MDA levels in high glucose exposed HK-2 cell lines. Results are expressed as mean ± SD (n=3) and the difference between the groups were evaluated by one – way ANOVA followed by Tukey's Post-hoc test. $^{**}p < 0.05$, $^{****}p < 0.05$ vs control, #### $p < 0.05$ vs HG.

Role of IPG treatment on lipid peroxidation

In HK-2 cells, exposure to HG resulted in a marked elevation of MDA content compared to the normal control, indicating enhanced oxidative lipid damage. Treatment with IPG (30µM) significantly attenuated this increase, demonstrating its efficacy in mitigating lipid peroxidation (Fig. 4).

Effect of IPG on the levels of ROS in HG-treated HK-2 cells

The levels of ROS in HG-induced HK-2 cells were significantly increased when compared to normal control. Treatment with IPG (30 µM) significantly reduced intracellular ROS accumulation in HK-2 cells, indicating its protective role against renal injury through the attenuation of oxidative stress (Fig. 5).



A

B

Fig. 5. IPG inhibits ROS generation in HG-treated HK-2 cells.

A. Fluorescence images of ROS in HK-2 cells B. Relative fluorescence intensity of ROS in experimental cell lines. Data are expressed as mean ± SD (n = 3) and the difference between the groups were evaluated by one-way ANOVA followed by Tukey's Post hoc test $^{*}p < 0.05$, $^{****}p < 0.05$ vs control, #### $p < 0.05$ vs HG.

Effect of IPG on Nrf2 expression in experimental cells

A significant downregulation in the expression of Nrf2 was observed in HG-treated HK-2 cells. On exposure with IPG (30μM),the expression was significantly upregulated (Fig. 6).

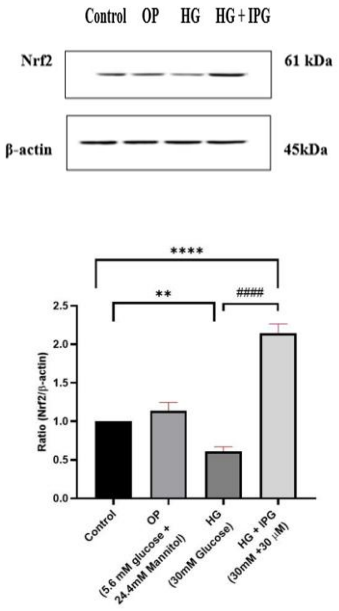


Fig. 6. Effect of IPG on Nrf2 expression in HK-2 cells. The expressions of Nrf2 was quantified by western blot. β-actin was used as the loading control. Data are expressed as mean ± SD (n=3) and the difference between the groups were evaluated by one-way ANOVA followed by Tukey's Post hoc test $^{**}p < 0.05$, $^{****}p < 0.05$ vs control, #### $p < 0.05$ vs HG.

DISCUSSION

Diabetic nephropathy (DN) a common microvascular complication of diabetes mellitus is marked by sustained hyperglycaemia, hypertension, progressive albuminuria, and a decline in glomerular filtration rate, all of which contribute to cardiovascular associated mortality (Yang *et al.*, 2017). DN affects approximately one-third of patients with diabetes mellitus (DM), with a ten-year cumulative mortality rate reaching 31.1% (Yao *et al.*, 2022). The development and progression of DN arise from a complex interplay of metabolic, hemodynamic, inflammatory and epigenetic factors. Among these interconnected mechanisms, OST is recognized as a central pathogenic factor (Wang and Zhang, 2024). Therefore, targeting OST and its downstream molecular mediators represents a critical therapeutic approach for mitigating renal damage in DN and slowing disease progression (Zhao and Wang, 2023; Li *et al.*, 2021).

The current investigation is focused on evaluating the *in vitro* protective effects of IPG against DN utilising HG-induced alterations in HK-2 cells. Previous studies utilizing natural compounds such as Psoralen and Paeonol in HK-2 cell models have indicated that treating cells with high glucose (30 mM) for 48 h provides an optimal condition for establishing glucose-induced cytotoxicity (Zhang *et al.*, 2018; Lin *et al.*, 2020). Our findings demonstrated that elevated glucose concentrations exert pronounced cytotoxic effects on HK-2 cells. To evaluate the protective efficacy of IPG under these conditions, both MTT and CCK-8 assays were performed. The data revealed that IPG effectively attenuated high glucose-induced cytotoxicity, thereby enhancing the viability of HK-2 cells.

Chronic hyperglycemia through mitochondrial dysfunction, activation of NADPH oxidase and AGE accumulation generates excess ROS (Hashemi *et al.*, 2023). This sustained oxidative burden disrupts the redox homeostasis within renal cells (Forbes and Thorburn, 2018; Darenskaya *et al.*, 2023). Consequently, OST amplifies inflammatory signaling, triggers apoptotic pathways in tubular epithelial cells and promotes extracellular matrix deposition, all of which contribute to progressive glomerular and tubulointerstitial injury (Zhan *et al.*, 2021; Xue *et al.*, 2024). In this study, HG conditions led to a marked increase in both ROS generation and level of MDA in HK-2 cells. However, treatment with IPG (30μM) significantly attenuated ROS and MDA levels suggesting its protective effects against proximal tubular injury in DN, which corroborates with Wang *et al.* (2019) who demonstrated that treatment with pyridoxamine reversed HG-induced OST in HK-2 cells.

Clinical evidence indicates that DN increases ROS production and impairs antioxidant defense mechanisms by altering the activity of antioxidant status (Uddandrao *et al.*, 2019).

SOD acts as a primary line of defense against ROS by converting them into less reactive intermediates (Kehrer and Klotz, 2015). Subsequently, hydrogen peroxide generated in this process is further detoxified by catalase (CAT), glutathione (GSH) and GPx. CAT, a heme-containing enzyme, exerts protection by limiting the formation of hydroxyl radicals, thereby safeguarding cellular components from peroxisome-induced OST. GPx, a selenium-dependent enzyme, reduces lipid hydroperoxides and free hydrogen peroxide to water through the oxidation of reduced glutathione (Tong and Shan, 2019). Glutathione S-transferases (GSTs) comprise a large enzyme superfamily involved in cellular detoxification and cytoprotection (Tesauro *et al.*, 2015). GSH, the predominant intracellular antioxidant, eliminates hydrogen peroxide and other peroxides via its thiol group derived from cysteine residues (Zuo *et al.*, 2018). In high glucose (HG) conditions, uncontrolled ROS production impairs antioxidant status, resulting in oxidative damage to biomolecules. Reports indicate that chronic hyperglycemia resulted in elevated OST causing damage to tissues (Lin *et al.*, 2020). However, treatment with IPG markedly improved the antioxidant status in HG-exposed HK-2 cells. Specifically, IPG enhanced SOD, CAT, GPx, GST activities and the level of GSH, while concurrently reducing lipid peroxidation, indicating its potential to counteract glucose-induced OST and preserve renal cell function. It has been documented that ALA/LA attenuated oxidative stress and apoptosis through ameliorating ROS/p38/TGF-β1 pathway in HK 2 cells (Jiang *et al.*, 2017).

Therapeutic strategies targeting Nrf2 signaling represent a promising avenue for intervention in the treatment of DN (Hernandez *et al.*, 2022). Nrf2 functions as a critical regulator of antioxidant gene expression, controlling the transcription of enzymes essential for maintaining cellular redox homeostasis (Bellezza *et al.*, 2018; Tonelli *et al.*, 2018). In the present study, exposure to HG markedly downregulated Nrf2 expression, which was significantly restored to near normal in IPG treated experimental cell lines.

Consistent with our findings, Astragaloside IV (AS-IV) significantly upregulated Nrf2 levels in high-glucose exposed HK-2 cells (Wang and Guo, 2019). Studies have highlighted that activation of Nrf2 is a potential strategy for DN treatment (Zhang *et al.*, 2018; Lu *et al.*, 2019).

CONCLUSION

From this we conclude that IPG decreased ROS production and curtailed OST thereby exhibiting its protective action against HG- induced renal tubular damage in HK-2 cells. These actions are mediated by activation of Nrf2 signaling, enhancement of antioxidant status under IPG treated conditions. Hence, IPG may be a promising therapeutic agent in alleviating DN.

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