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Effect of *Orthosiphon stamineus* Extract on HIF-1A, Endothelin-1, and VEGFR-2 Gene Expression in NRK-52E Renal Tubular Cells Subjected to Glucotixicity

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Abstract: This study aimed to investigate the impact of *Orthosiphon stamineus* extract on gene expression in NRK-52E cells under conditions of glucotoxicity. Gene expression analysis using RT-PCR was conducted following exposure of cultured NRK-52E cells to glucotoxic conditions and various concentrations of *Orthosiphon stamineus* extract. The results revealed a dose-dependent decrease in HIF-1α, ET-1 and VEGFR-2 gene expression levels upon treatment with *Orthosiphon stamineus* extract. The diverse array of phytochemicals found within *Orthosiphon* demonstrates a significant impact on the biochemical pathways implicated in the pathogenesis of the disease. Additionally, molecular docking studies suggested the potential inhibition of Rho-kinase as a mechanistic explanation for this observed effect. These findings suggest that *Orthosiphon stamineus* extract may possess renoprotective properties against glucotoxicity-induced cellular damage. This study contributes to understanding potential therapeutic interventions for managing renal complications associated with conditions such as diabetes.

Introduction

Orthosiphon stamineus Benth. (Lamiaceae) is a perennial medicinal herb cultivated in tropical countries (Shafaei et al., 2017). It has been used to manage diabetes mellitus and its complications, particularly chronic renal failure and diabetic nephropathy (Sun et al., 2014). Various phytochemicals present in *Orthosiphon stamineus* effectively reverse diabetic complications by acting through various pathogenic pathways. Arjunolic acid is known to reduce the Nuclear Factor Kappa-B (NF-Kb) in glomeruli (Manna and Sil, 2012). Ursolic acid reduces advanced glycosylation end products, tumor necrosis factor-α, and interleukin-1 and increases the expression of superoxide dismutase in cardiac muscle (Wang et al., 2018). Ursolic acid also decreases NF-

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kB and Janus kinase (JNK) activity (Xu et al., 2018). In this study, we aim to evaluate the expression of important genes involved in the pathogenesis of diabetic nephropathy. In hyperglycemia, Hypoxia-inducible factor-1α causes apoptosis in renal tubules by inducing Heme oxygenase (HO)-1, a gene (Ferroptosis) (HIF-1α) (Sun et al., 2012; Chiang et al., 2018; Feng et al., 2021; Song et al., 2022). Elevated HIF activity in diabetes is detrimental to renal glomeruli and tubular cells (Rosenberger et al., 2008; Iso et al., 2010; Patrik Persson, 2017). Plasma and urinary Endothelin-1 levels were elevated in patients with diabetic nephropathy and antagonizing ET1 receptors were shown to be beneficial to diabetic kidneys (Peppa-Patrikiou et al., 1998; Wenzelet et al., 1999). A VEGFR2 pathway-activation blockade improved renal function and alleviated glomerular damage in diabetic mice models (Lavoz et al., 2020). GSK-3b plays a detrimental role and contributes to the pathogenesis of diabetic kidney disease, and GSK-3b inhibition can improve kidney function in diabetics (Lin et al., 2006; Paeng et al., 2014; Guo et al., 2016; Pawar et al., 2023; Sarkar et al., 2023; Biswas et al., 2023). Matoba and Keiichiro, (2009) have shown that Rho-kinase inhibition prevents the progression of diabetic nephropathy by downregulating hypoxia-inducible factor 1α. In this experiment, we intend to study the effect of *Orthosiphon* extract on the gene expression of Endothelin-1, VEGFR-2, and HIF-1 alpha in NRK-52E (rat renal proximal tubular epithelial cell) cells subjected to glucotoxicity and also study the docking properties of the phytochemicals of *Orthosiphon* on GSK-3b and Rho-kinase, which regulate HIF-1α levels.

Materials and Methods

Plant collection and preparation of crude extract

Fresh and healthy plants of *Orthosiphon stamineus* were procured from its natural habitat in Siruthavur, Chengalpattu district, Tamil Nadu, India. Fresh leaves were washed and shade-dried for 3 days at room temperature. The dried leaves were powdered into fine particles with the help of a blender (Venus, India) and stored in a sterile airtight container until further use. Fifty grams (50g) of powdered plant sample were extracted with 1000 mL of 99% methanol in a Soxhlet apparatus. The extract was concentrated to dryness using a rotary evaporator. Twenty mg of the concentrated extract was dissolved in 1 ml of dimethyl sulfoxide (DMSO) and used as the stock solution, from which different concentrations were used for the treatment of cell lines.

Cell line and maintenance

NRK-52E cells, obtained from the National Centre for Cell Science in Pune, India, were cultured in Dulbecco's modified Eagle's medium (DMEM). The culture medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL amphotericin, and 3.7 g/L sodium bicarbonate. Culturing was conducted in a controlled environment at 37°C in a 5% CO_2 atmosphere. Harvesting of cells was performed when confluence reached 85 to 90%, and subsequent seeding after trypsin treatment was at a density of $1x10^6$ cells. For cell treatment with plant extract, a duration of 24 hours was employed. This protocol ensured the maintenance and treatment of NRK-52E cells under optimal conditions for experimental purposes.

NRK 52E Cell Treatment with *Orthosiphon Stamineus* Extracts

The experimental conditions involved the exposure of cells to various glucose concentrations and different concentrations of plant extract. The glucose concentrations tested included low glucose at 5.5 mM (1 g/L) and high glucose at 30 mM (4.5 g/L). In addition to these, cells were subjected to high glucose conditions of 30 mM (4.5 g/L) in combination with varying concentrations of plant extract, specifically 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, and 400 µg/ml. These conditions aimed to assess the effects of different glucose levels and plant extract concentrations on cellular responses. Such controlled variations in glucose and extract concentrations allow for systematically investigating potential interactions and effects on cellular behaviour. This experimental design facilitates the exploration of potential therapeutic or detrimental impacts of the plant extract under different glucose environments, providing valuable insights into its potential utility in modulating cellular responses associated with glucose levels.

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RNA isolation

RNA isolation was done from cells using TRIZOL reagent by standard lab protocol involving centrifugation (12,000×g) for 20 minutes at 4°C and precipitation by isopropanol. Nano drops RNA quantification done. The cDNA library was constructed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Real-time PCR analysis

The real-time qPCR was conducted for SIRT1, HIF-1α, ET-1, and VEGFR-2 using the SYBR green qPCR mastermix (Takyon, Eurogentec).

HIF-1α:

Forward Primer: 5′GCCGCTGGAGACACAATCAT3′
Reverse Primer: 5′GAAGTGGCTTTGGCGTTTCA 3′

ET-1:

Forward Primer:5′CAGGGCTGAAGACATTATGGAGA3′
Reverse Primer: 5′CATGGTCTCCGACCTGGTTT3′

VEGFR-2:

Forward Primer: 5′CGGACAGTGGTATGGTTCTTGC3′,
Reverse Primer; 5′GTGGTGTCTGTGTCATCGGAGTG3′

The PCR amplification was performed using the following thermal cycler program. Initial denaturation at 95°C for 3 minutes, denaturation 95°C for 10 seconds, primer annealing at 60°C for 20 seconds and extension at 72°C for 1 minute. The cycle threshold (CT) values obtained were analyzed by $2^{-\Delta\Delta CT}$ formula.

Docking Study

The docking protocol utilized in this study involved the utilization of ligands obtained from the PubChem database, totaling 15 ligands. The protein structures utilized for the docking simulations included GSK-3b (PDB code: 6hk3) and Rho-kinase (PDB code: 2h9v), acquired from the RCSB Protein Data Bank. AutoDockVina served as the primary docking software, facilitating the computational docking simulations. These docking experiments were conducted using the PyRx platform, which provided a user-friendly interface for efficient setup and management of the docking runs. Additionally, for the visualization of hydrogen bond interactions between the ligands and the amino acid residues of the protein, the PYMOL software was employed, offering insights into potential binding modes and affinities at the molecular level.

Table 1 : Docking protocol

Ligands	Downloaded from Pubchem site. 15 ligands
Protein structures	GSK-3b (6hk3), RHO-kinase (2h9v) downloaded from RCSB PDB (PW.Rose 2017)
Docking Software	AutoDock Vina (OT.Trott 2010)
Docking platform	PyRx
Amino-acid Hydrogen bond visualization platform	PYMOL (De Lano2002)

Results

Quantitative expression analysis HIF-1α Gene

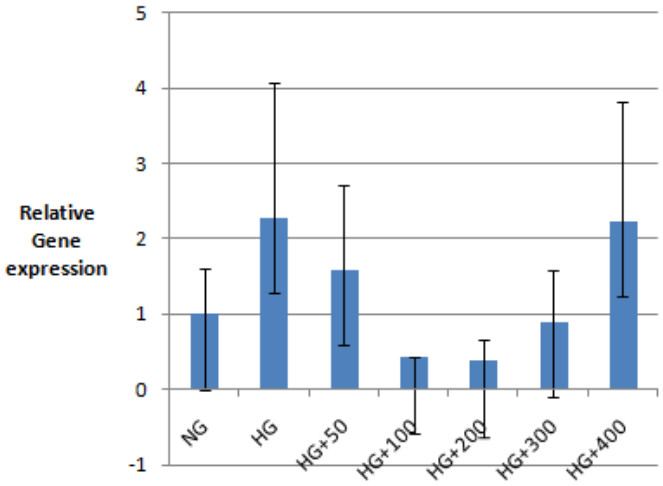

Figure 1. Expression of HIF-1α in NRK-52E treated with *Orthosiphon stamineus* plant extract under low and high glucose conditions. NG: normal glucose; HG: high glucose; 100, 200, 300, 400: concentration of plant extract in µg/ml

There was a 2.2794-fold increase in the expression of the HIF-1 gene when the cells were exposed to high glucose compared to those exposed to a normal glucose level. However, the expression of the HIF-1 gene decreased after treatment with different concentrations of plant extract in a dose-dependent manner up to 200 µg/ml. The decrease in the expression level was found to be in the range of 1.59–0.37 fold when the cells were treated with 50–200 plant extract. The lowest expression of the gene was observed in the cells treated with 200 µg/ml plant

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extract. The cells treated with 300 µg/ml of plant extract showed reduced expression (0.89±0.79) of the gene when compared to the control however was found to be relatively higher when compared to the cells treated with 200 µg/ml. At 400 µg/ml, the therapeutic benefit was lost and again the gene expression increased to glucotoxic levels (2.23±1.58).

Quantitative expression analysis Endothelin-1 Gene

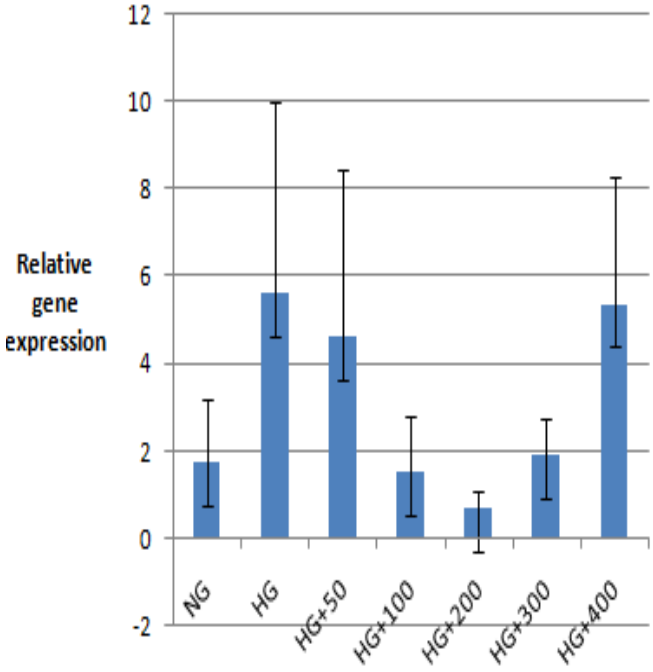

Figure 2. Expression of Endothelin-1 in NRK-52E treated with Orthosiphon stamineus plant extract under low and high glucose conditions. NG: normal glucose; HG: high glucose; 100, 200, 300, 400: concentration of plant extract in µg/ml.

The ET-1 gene expression was also found to be similar to the HIF-1α. There was a 5.64± 5.34 fold increase in the expression of the ET-1 gene in the cells that were grown under the high glucose condition. Treatment with different concentrations of *Orthosiphon stamineus extracts* counteracted the increase in ET-1 expression in a dose-dependent manner, starting from 50 µg (4.64±3.83) to 200 µg/ml (0.70±0.37). The lowest gene expression level was observed with 200 µg/mL cells. At 300µg/ml, the expression was 1.91±0.81, still lower than hyperglycemia-induced elevation in gene expression. At 400 µg/ml, the therapeutic benefit was lost, and again, the gene expression increased to glucotoxic levels (5.38±2.98).

Quantitative expression analysis VEGFR-2 Gene

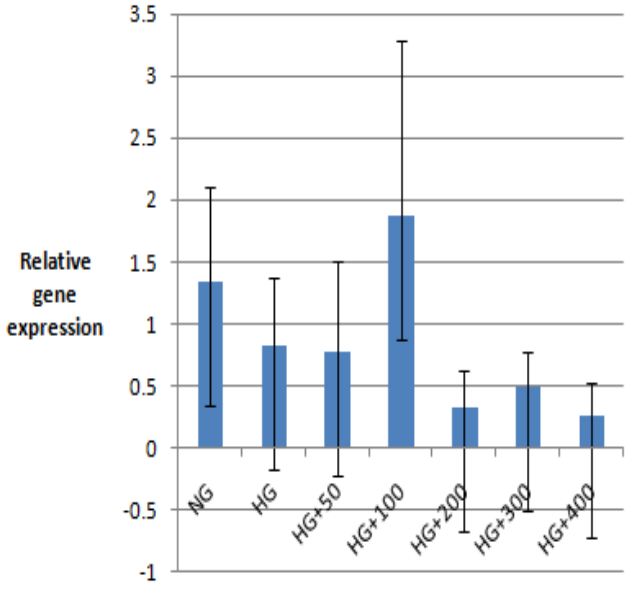

Figure 3. Expression of VEGFR-2in NRK-52E treated with *Orthosiphon stamineus* plant extract under low and high glucose conditions. NG: normal glucose; HG: high glucose; 100, 200, 300, 400: concentration of plant extract in µg/ml.

In the case of the VEGFR-2 gene, glucotoxicity decreased the gene expression levels compared to normoglycemia, and treatment with Orthosiphon extract did not show any consistent increase or decrease in gene expression levels. However, at concentrations above 100 µg/ml, there was a consistent reduction in VEGFR-2 gene expression up to 400 µg/ml.

Docking Study

Results

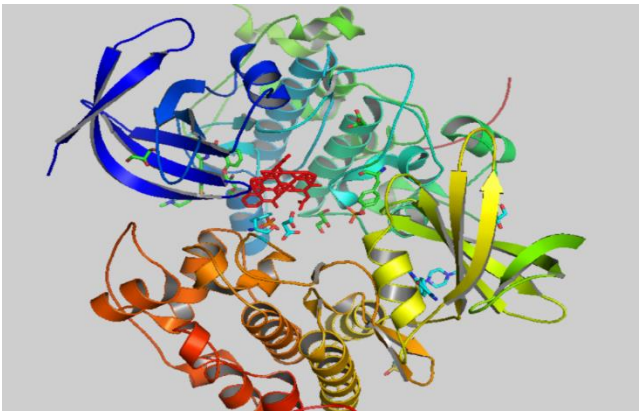

Figure 4. Molecular docking analysis of GSK-3b protein (6hk3) with staminol-D in the active G8B native ligand binding site.

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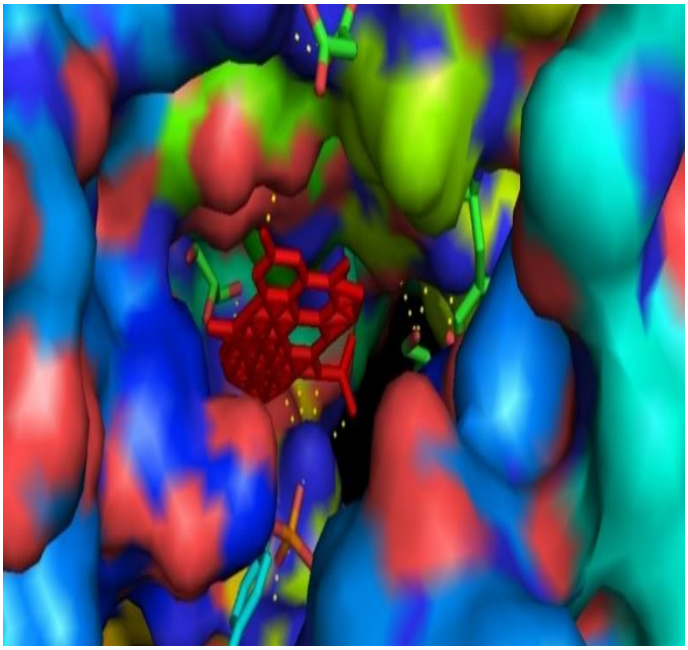

Figure 5. Molecular docking analysis of GSK-3b protein (6hk3) with staminol-D in-active G8B native ligand binding site.

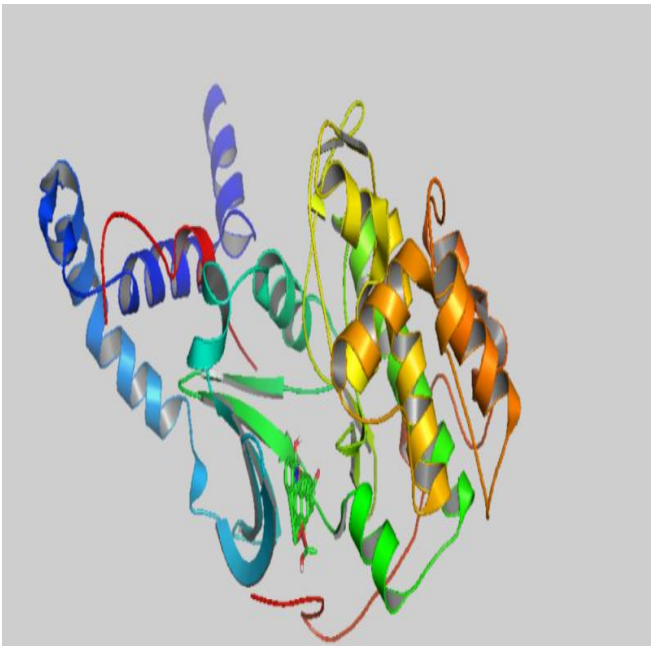

Figure 6. Molecular docking analysis of RHO-kinase structure (2h9v) with Secorthosiphol-Cin-active G8B native ligand binding site.

Table 2. Docking score of phytochemicals found in the extract of *Orthosiphon stamineus* to GSK-3b protein (6hk3) structure

Sl. No.	Ligands	Docking score with GSK-3B protein	Participating amino acid(s) of the protein
1	Reference ligand	-8.4(G8B)	Ser-208,Ser-66
3	Norstaminol_B	-9.7	Lys-197
4	Orthosiphol_J	-12.1	Arg-141
5	Orthosiphol_M	-12.7	Arg-220,Ser-203
7	Orthosiphol_V	-11.3	Arg-220,Gln-185
8	Orthosiphol_Z	-9.4	Thr-253
10	Secoorthosiphol_B	-9.7	Arg-141, Arg-144
11	Secoorthosiphol_C	-9.7	Arg-220, Asp-200
12	Sinensetin	-6.4	Ser-203,Gln-185
13	Staminol_D	-9.7	Ser-203, Arg-220, Ser-219
14	Scuttelarein	-6	His-145, Arg-148, Arg-141
15	Vomifoliol	-6.5	Gly-65, Ser-66

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Table 3. Docking score of phytochemicals found in the extract of *Orthosiphon stamineus* to Rho-kinase protein (2h9v)

Sl. No.	Ligands	Docking score with Rho-kinase protein (2h9v)	Participating amino acid(s) of the protein
1	Reference ligand	-6.3(Y27)	Asp-176, Asn-219, Asp-232
2	Ladanein	-6.6	Asn-179
3	Norstaminol_B	-11.4	Asn-179
4	Orthosiphol_J	-11.7	Asp-385
5	Orthosiphol_M	-10.4	Asp-385
7	Orthosiphol_V	-10.3	Asp-385
8	Orthosiphol_Z	-9.6	Thr-253
11	Secoorthosiphol_C	-10.8	Asp-385,Asp-386
13	Staminol_D	-10	Asp-385
15	Vomifoliol	-6.9	Asp-385, Ile-98

Discussion

The gene expression studies show that *Orthosiphon stamineus extracts* counteracted the increase in HIF-1 expression in the dose range of 50µg/ml to 300 µg/ml. Similar action was also seen in the gene expression of endothelin-1. As far as we know, this is the first report on *Orthosiphon stamineus* extract reducing gene expression of HIF-1 and endothelin-1 in renal tubular cells. Further docking studies have shown that norstaminol_b, orthosiphol_j, orthosiphol_m, orthosiphol_v, orthosiphol_n, orthosiphol_z, staminol_d, secoorthosiphol_c, and secoorthosiphol_bhas a higher docking score compared to native ligands of the protein structures GSK-3b and Rho-kinase. Rho-kinase inhibition prevents the progression of diabetic nephropathy by downregulating hypoxia-inducible factor 1α (Matoba, 2009). As *Orthosiphon* phytochemicals show a good docking score, their binding may result in Rho-Kinase inhibition and, thereby down regulation of HIF-1α. As inhibition of GSK-3b can sometimes upregulate HIF-1α, the HIF-1α downregulation seen with *Orthosiphon* may be due to Rho-kinase binding and inhibition rather than via GSK-3b. In addition, due to its high docking score with GSK-3b, *Orthosiphon* can also have GSK-3b inhibitory activity, which may also be beneficial for the treatment of diabetic kidney disease. However, at higher concentrations, the benefits of the extract are lost, probably due to the direct toxicity of the extract.

Conclusion

Orthosiphon plant has multiple phytochemicals that influence the biochemical pathways involved in the pathogenesis of diabetic nephropathy, making it a promising treatment option for the disease. In conclusion, the comprehensive analysis of the *Orthosiphon* plant highlights its potential therapeutic efficacy in addressing diabetic nephropathy. The diverse array of phytochemicals found within *Orthosiphon* demonstrates a significant impact on the biochemical pathways implicated in the pathogenesis of the disease. This underscores the plant's promising role as a treatment option for diabetic nephropathy, offering avenues for further exploration and development in the field of natural medicine.

Conflict of Interest

The authors declare no conflict of interest.

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