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Nano formulated Hesperidin Modulates the PI3K/mTOR Signalling Pathway: A Potential Therapeutic Approach for Diabetic Nephropathy in a Rat Model

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Abstract

Diabetic nephropathy, a chronic and incapacitating kidney disease stemming from diabetes mellitus, poses a significant health concern. This research aimed to assess the effectiveness of nano formulated hesperidin as a potential therapeutic intervention for diabetic nephropathy, utilizing a rat model. The study delved into the intricate molecular mechanisms underlying hesperidin's impact, focusing on its modulation of the PI3K/mTOR signaling pathway. Kidney function tests, including urea and creatinine, revealed reduced levels, indicative of hesperidin's potential renoprotective effects. Western blot analysis exhibited a downregulation of PI3K and mTOR protein expression, further corroborating the therapeutic influence of nano formulated hesperidin. Concurrently, gene expression analysis via RT-PCR demonstrated a consistent reduction in PI3K and mTOR transcript levels in kidney samples from rats treated with nano formulated hesperidin. Molecular docking analysis provided additional insights, revealing a robust binding affinity between hesperidin and the PI3K/mTOR targets. This holistic approach, combining biochemical, molecular, and computational analyses, strengthens the proposition that nano formulated hesperidin holds promise as an efficacious therapeutic strategy for diabetic nephropathy. The observed reductions in kidney function markers, protein expression, and gene transcription collectively underscore the potential of nano formulated hesperidin in mitigating the deleterious effects of diabetic nephropathy, offering new avenues for targeted and effective treatment.

Keywords: Diabetic nephropathy, Nano formulated hesperidin, PI3K/mTOR signalling pathway, Rat model, Kidney function tests, Molecular docking, Therapeutic intervention.

Introduction

Diabetic nephropathy (DN) is a chronic and debilitating kidney disease that manifests as a complication of diabetes mellitus. DN is a prominent contributor to end-stage renal disease on a global scale, impacting individuals diagnosed with both type 1 and type 2 diabetes. The development of diabetic nephropathy is closely associated with prolonged high levels of blood sugar, setting off a series of interconnected physiological processes within the kidneys [1]. Hyperglycaemia leads to glomerular hyperfiltration, renal hypertension, and subsequent thickening of the glomerular basement membrane. This process results in increased permeability, renal hypoperfusion, and proteinuria, with albumin being a predominant excreted protein. Over time, chronic inflammation and oxidative stress contribute to renal fibrosis, impairing kidney function [2]. Clinical features encompass microalbuminuria, evolving into proteinuria, hypertension, edema, and a decline in glomerular filtration rate. Diagnosis involves urinalysis, serum creatinine assessment, and monitoring of glomerular filtration rate. Management strategies emphasize tight glycaemic control, blood pressure management with medications like ACE inhibitors or ARBs, lifestyle modifications, and regular monitoring [3]. Early detection through routine screening and proactive management of risk factors play pivotal roles in preventing or delaying the onset of diabetic nephropathy, underscoring the importance of comprehensive care in individuals with diabetes. The current treatment strategies for diabetic nephropathy aim to manage the progression of renal damage and mitigate complications associated with this serious complication of diabetes [4]. Tight glycaemic control stands as a cornerstone, with the optimization of blood glucose levels playing a pivotal role in slowing the advancement of nephropathy [5]. These medications not only help control hypertension but also contribute to reducing proteinuria, thus preserving kidney function. Lifestyle modifications, including a renal-friendly diet, weight management, and regular exercise, complement pharmacological interventions. In cases of advanced diabetic nephropathy, renal replacement therapies such as dialysis or kidney transplantation may be considered. Monitoring kidney function, blood pressure, and glycaemic levels through regular assessments is paramount for the ongoing management of diabetic nephropathy [6]. Research continues to explore novel therapeutic approaches, emphasizing the need for a comprehensive and individualized treatment plan to address the multifaceted nature of this condition. Early detection, timely intervention, and a holistic approach to care remain fundamental in improving outcomes and enhancing the quality of life for individuals affected by diabetic nephropathy [7]. Utilizing nano-formulated natural drugs presents a pioneering strategy in combating diabetic nephropathy, exploiting the benefits of nanotechnology to amplify the therapeutic effectiveness of bioactive compounds sourced from nature [8]. The unique properties of nanomaterials, such as increased surface area and improved bioavailability, contribute to their potential in addressing the multifaceted aspects of diabetic nephropathy. Nano formulations enable targeted drug delivery, minimizing systemic side effects and enhancing drug accumulation at the site of kidney damage [9]. Several natural compounds with anti-inflammatory, antioxidant, and anti-fibrotic properties have shown promise in mitigating diabetic nephropathy [10-12]. For instance, curcumin from turmeric [13], resveratrol from grapes [14], and quercetin from fruits [15] and vegetables exhibit renoprotective effects. However, their therapeutic potential is often hindered by limited solubility and poor bioavailability. Nano formulations overcome these challenges by encapsulating the natural drugs within nanoparticles, improving their stability

and allowing sustained release. Studies have demonstrated that Nano formulated natural drugs can attenuate oxidative stress, inflammation, and fibrosis in diabetic nephropathy models [16]. These formulations exhibit enhanced cellular uptake and bioactivity, leading to improved outcomes in terms of kidney function and histopathological changes. Additionally, the targeted delivery of these Nano formulations to renal tissues reduces off-target effects on other organs [17].

The development of Nano formulated natural drugs involves various techniques, including nanoparticle synthesis, encapsulation, and surface modification [18]. These approaches enable the customization of nanoparticles to optimize drug release kinetics and enhance therapeutic effects. Despite promising preclinical results, the translation of Nano formulated natural drugs to clinical applications requires further investigation. Challenges such as long-term safety, scalability, and regulatory considerations need to be addressed. Nevertheless, the evolving field of nanomedicine holds substantial potential for advancing the treatment of diabetic nephropathy by harnessing the therapeutic properties of natural compounds in a targeted and efficient manner. In this research endeavour, our primary objective was to examine the efficacy of Nano formulated hesperidin in addressing diabetic nephropathy within a rat model. The study focused on elucidating the potential therapeutic impact of Nano formulated hesperidin and its modulation of the PI3K/mTOR signaling pathway.

Materials and Methods

Chemicals and reagents

Chemicals and reagents sourced from Sigma, Cell Signaling Technology, Santa Cruz Biotechnology, Sigma Chemical Company, Krishgen Biosystems, and Invitrogen were utilized in this research. The RNA isolation kit employed was obtained from Invitrogen, and the study utilized primers provided by Eurofins Genomics India Pvt. Ltd.

Animals and Treatment grouping

Male albino Wistar rats, aged 80 days and weighing between 180 and 200 g, were housed in controlled conditions at BRULAC, SDC, SIMATS, Tamil Nadu, India. These rats were fed standard rat pellets and had unrestricted access to clean water. Following established protocols, the study spanned 4 weeks, during which rat groups were induced with STZ. The experiments involving animals were conducted following approval from the relevant regulatory body. Male rats, aged eight weeks and weighing g, were procured and housed in appropriate environmental conditions, with access to standard laboratory diet and water ad libitum. On day 58, fasting blood sugar levels were measured after an overnight fast, identifying rats with levels exceeding 120 mg/dL as having T2DM. Throughout the trial, the rats consistently consumed a high-fat diet supplemented with sucrose.

Group 1: Control rats, Group 2: Diabetic rats induced, Group 3: Diabetic rats treated with 6 mg/kg b. wt of Glibenclamide for 45 days, Group 4: Diabetic rats treated with 100 mg/kg b. wt NF-HSP for 45 days, Group 5: Control rats treated with 100 mg/kg b. wt NF-HSP alone for 45 days. Twenty-four-hour urine samples were collected from the rats, and blood pressure was measured using the tail-cuff method. Various glycemic indices, including body weight, glucose levels (mg/dl), insulin levels, and HbA1c, were assessed.

Kidney Function Tests

Serum creatinine (mg/dl), serum urea (mg/dl), blood urea nitrogen (BUN) (mg/dl), and urine albumin (mg/dl) concentrations were measured according to standardized procedures [19]

utilizing an autoanalyzer (Hitachi 7600, Japan). These parameters were assessed to evaluate renal function and detect potential abnormalities in the experimental subjects.

Anti-oxidant activity

Oxidative stress markers, including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase (GPx), were assessed following established protocols [20]. These markers provide insights into the oxidative status and antioxidant defence mechanisms within the experimental subjects, aiding in the evaluation of oxidative stress levels and antioxidant capacity.

Gene expression by RT-PCR

The extraction of total RNA from both control and experimental samples was performed using a TRIR kit (Total RNA Extraction Reagent) from Abgene, UK. Quantification of the extracted RNA was conducted via spectrophotometry, following the methodology outlined by [21], with measurements expressed in micrograms (µg). Subsequently, cDNA synthesis was conducted using 2 µg of total RNA utilizing the reverse transcriptase kit from Eurogentec (Seraing, Belgium), following the manufacturer's instructions. Real-time PCR was carried out using the Takara SyBr Green Master Mix along with specific forward and reverse primers listed in Table 1. Positive and negative controls consisted of control DNA and water, respectively. The resulting data were visualized using the CFX96 Touch Real-Time PCR detection system, USA.

Protein expression by Western blot

The kidney tissues were harvested, homogenized, and centrifuged at 4°C. Subsequently, 50 µg of protein samples were prepared for blotting, employing antibodies targeting IL6, TNF-α, and IL-1 (dilution: 1:1000, sourced from CST, USA). The intensity of the corresponding bands was analyzed using ImageJ software and standardized against the reference value provided by β-actin. This method facilitated the quantification of protein expression levels for the selected inflammatory markers in the kidney tissues.

Molecular docking analysis

The research delves into investigating the binding interactions between hesperidin (CID: 10621) and various proteins, namely mTOR (4JSV), Glut4 (4POO), and AS160 (2WW8) whose structures were obtained from the Protein Data Bank (PDB) at https://www.pdb.org/pdb. The docking analysis was conducted using PyRx software. Following this, both 3D and 2D structural docking analyses were visualized and scrutinized utilizing Discovery Studio 2021.

Statistical analysis

The study reported all findings as the mean ± standard deviation derived from six rats in each experimental group. Statistical analysis was carried out utilizing One-way analysis of variance (ANOVA) conducted with GraphPad Prism 8 software. To assess differences between groups, pairwise comparisons were conducted using the least significant difference test, where significance was established at $p<0.05$. This comprehensive statistical approach facilitated the robust evaluation of intergroup variations and allowed for the determination of significant differences in the experimental outcomes.

Results

Nano-formulated hesperidin exhibits biochemical indices in DN rats

Four weeks following STZ injection, diabetic rats showed an increase in body weight compared to the control group. However, administration of nano-formulated hesperidin led to a reduction

in both body weight and glucose levels. Moreover, diabetic-induced rats showed a decrease in albuminuria levels, which were subsequently elevated upon treatment with nano-formulated hesperidin. Additionally, urine volume and glycemia levels were found to be heightened in diabetic animals but were attenuated in those treated with nano-formulated hesperidin, as delineated in Table 2. Comparatively, the glibenclamide treatment groups exhibited minimal variation in comparison to the nano-formulated hesperidin group. These findings initially suggested that nano-formulated hesperidin might induce alterations in diabetic nephropathy.

Renal function markers for nano-formulated hesperidin in DN rats

Diabetic-induced rats exhibited elevated levels of serum creatinine, serum uric acid, and BUN. However, the administration of nano-formulated hesperidin to diabetic rats resulted in significant improvements, leading to decreased levels of serum creatinine, serum uric acid, and BUN compared to the control group. The standard drug glibenclamide group also demonstrated a reduction in these renal function markers, as depicted in Fig. 1. Additionally, the levels of urea, creatinine, and creatinine clearance were reduced in the diabetic-induced group, whereas the nano-formulated hesperidin group exhibited increased levels compared to the control group. These findings suggest potential therapeutic effects of nano-formulated hesperidin on renal function in diabetic rats.

Nano-formulated hesperidin exhibits anti-oxidant activity in kidney of DN rats

Renal levels of MDA and Nitric Oxide (NO), indicators of lipid peroxidation, were observed to be elevated in diabetic-induced animals. However, the group treated with nano-formulated hesperidin exhibited reduced MDA levels. Additionally, oxidative enzymes such as SOD and their activity were measured, revealing decreased levels in the diabetic group compared to the control. Conversely, the nano-formulated hesperidin-treated group displayed increased levels of these enzymes compared to the control group, as depicted in Fig. 2. Furthermore, levels of GSH and TAC were found to be decreased in diabetic rats but increased upon treatment with nano-formulated hesperidin, surpassing levels observed in the control group. Overall, treatment with nano-formulated hesperidin in diabetic-induced rats resulted in reduced MDA and NO levels and increased levels of SOD, GSH, and TAC compared to the control group, indicating potential antioxidant effects.

Gene expression by RT-PCR

In our study, we aimed to further validate the potential of nano-formulated hesperidin in improving insulin sensitivity and glucose metabolism, potentially mediated by its effects on the PI3K signaling pathway. To achieve this, we conducted gene expression analysis by RT-PCR on kidney tissues to examine the molecular effects of nano-formulated hesperidin compared to diabetic-induced, control, and standard drug groups. Our results revealed that the expression levels of key genes involved in the PI3K signaling pathway, including mTOR, PI3K, Glut4, and AS160, were upregulated in the diabetic-induced kidney tissues. Remarkably, the group treated with nano-formulated hesperidin exhibited reduced expression levels of these genes, as depicted in Fig. 3. These findings strongly suggest that nano-formulated hesperidin treatment modulates the PI3K/mTOR signaling pathways in kidney tissues. This evidence highlights the potential of nano-formulated hesperidin as a therapeutic agent for regulating insulin sensitivity and glucose metabolism, possibly through its modulation of the PI3K signaling pathway. Further investigations are warranted to elucidate the underlying

mechanisms and potential clinical applications of nano-formulated hesperidin in the treatment of metabolic disorders such as diabetes.

Protein expression by Western blot

In continuation of our study, we conducted protein expression analysis by western blot on kidney tissues to investigate the molecular effects of nano-formulated hesperidin in comparison to diabetic-induced, control, and standard drug groups. Our findings revealed upregulated expression levels of key genes involved in the PI3K signaling pathway, including mTOR, PI3K, Glut4, and AS160, in the diabetic-induced kidney tissues. Notably, the group treated with nano-formulated hesperidin exhibited reduced expression levels of these genes, as illustrated in Fig. 4. These results strongly suggest that nano-formulated hesperidin treatment modulates the PI3K/mTOR signaling pathways in kidney tissues. This evidence underscores the potential of nano-formulated hesperidin as a therapeutic agent for regulating insulin sensitivity and glucose metabolism, likely through its modulation of the PI3K signaling pathway.

Molecular docking analysis

To further investigate our hypothesis that hesperidin regulates the activity of PI3K regulating targets, we conducted molecular docking studies. Our aim was to assess the interaction between hesperidin and key proteins involved in PI3K pathway, including mTOR (-10.4 kcal/mol), GLUT4 (-11.2 kcal/mol), and AS160 (-8.6 kcal/mol) as shown in fig. 5. Notably, hesperidin exhibited higher binding energy with mTOR, AS160, and Glut4, indicating a strong potential for interaction shown in Table 3. This suggests that hesperidin may directly interact with these proteins and potentially modulate their activity. Overall, the results from the molecular docking studies align with the observed effects of hesperidin potential. These findings suggest that hesperidine may interfere with the PI3K/mTOR signaling pathway, leading to diabetes. Further investigations are warranted to fully understand the molecular mechanisms underlying the interaction between hesperidin and these PI3K/mTOR regulating targets.

Discussion

Diabetic nephropathy, a severe complication of diabetes mellitus, stands as a leading cause of end-stage renal disease (ESRD) worldwide [2]. It predominantly afflicts individuals with both type 1 and type 2 diabetes, marked by progressive kidney damage ensuing from chronic hyperglycemia. Prolonged exposure to elevated blood glucose levels induces injury to the small blood vessels in the kidneys, known as glomeruli. This results in augmented deposition of extracellular matrix within mesangial cells, leading to mesangial expansion and glomerulosclerosis [4, 5]. With disease progression, the glomerular filtration barrier becomes increasingly permeable, culminating in the leakage of albumin and other proteins into urine, termed as albuminuria or proteinuria. Persistent inflammation, oxidative stress, and activation of profibrotic signaling pathways contribute to renal fibrosis, ultimately leading to irreversible kidney damage. The clinical manifestation of diabetic nephropathy varies depending on the disease stage [6], initially presenting with minimal symptoms that may progress as kidney function declines.

Diagnosis of diabetic nephropathy entails a multifaceted approach, combining clinical assessment, laboratory tests, and imaging studies. Urinary albumin-to-creatinine ratio (UACR) or protein-to-creatinine ratio (PCR) measurement is employed to assess albuminuria/proteinuria [7], while serum creatinine levels and estimated glomerular filtration rate (eGFR) calculation aid in evaluating renal function. Renal ultrasound or other imaging

modalities are utilized to evaluate kidney size, structure, and the presence of renal artery stenosis [11, 22]. Over the years, extensive research has been devoted to exploring potential therapeutic interventions for managing diabetic nephropathy, encompassing both pharmacological and non-pharmacological approaches [23]. An emerging area of interest in diabetic nephropathy management is phytotherapy, which harnesses plant-derived compounds for therapeutic purposes. Phytotherapy has garnered significant attention owing to its perceived efficacy, safety, and relatively low cost compared to conventional medications [24].

Nano-formulated natural compounds hold significant importance in diabetes management due to their enhanced bioavailability, improved pharmacokinetics, and targeted delivery. These compounds, derived from natural sources such as plants, exhibit various bioactive properties that can effectively address different aspects of diabetes pathophysiology [25]. For instance, compounds like curcumin, resveratrol, quercetin, and berberine have demonstrated anti-inflammatory, antioxidant, and antidiabetic effects [26].

Nano formulation enhances the solubility and stability of these natural compounds, enabling better absorption and distribution in the body. This can lead to improved efficacy in controlling blood glucose levels, reducing insulin resistance, and protecting pancreatic beta cells [27]. Additionally, nano-formulated natural compounds can target specific cellular pathways involved in diabetes, such as the PI3K/Akt pathway, AMPK activation, or inhibition of inflammatory mediators like NF-kB. Furthermore, nano-formulated natural compounds may have fewer side effects compared to conventional medications, making them attractive options for long-term diabetes management [28]. They can also be combined with existing therapies to enhance their effectiveness or mitigate side effects. Hesperidin, a flavonoid found abundantly in citrus fruits, possesses potent antioxidant and anti-inflammatory properties. These properties help in combating oxidative stress and inflammation, which are key contributors to the development and progression of diabetes [29, 30]. Its antioxidant and anti-inflammatory properties, along with its ability to improve vascular function, may contribute to this protective effect [31]. This allows for lower doses to achieve therapeutic effects, reducing the risk of side effects and enhancing patient compliance. Nano-formulation enables targeted delivery of hesperidin to specific tissues or cells involved in diabetes pathology, such as pancreatic beta cells or insulin-sensitive tissues like muscle and adipose tissue [32]. This targeted approach enhances the therapeutic efficacy of hesperidin while minimizing off-target effects.

In this study, diabetic rats, induced by streptozotocin (STZ) injection, exhibited increased body weight, elevated glucose levels, and heightened renal dysfunction markers compared to the control group. However, administration of nano-formulated hesperidin led to a reduction in body weight, glucose levels, and improved renal function. Specifically, nano-formulated hesperidin treatment resulted in decreased levels of serum creatinine, serum uric acid, and BUN, along with an increase in urea, creatinine, and creatinine clearance compared to controls. Additionally, diabetic rats displayed elevated levels of renal lipid peroxidation markers, MDA, and Nitric Oxide (NO), along with decreased activity of antioxidant enzymes such as SOD. Treatment with nano-formulated hesperidin mitigated these effects, resulting in reduced MDA and NO levels, and increased SOD, GSH, and TAC levels compared to controls. These findings suggest that nano-formulated hesperidin may have therapeutic potential in ameliorating diabetic nephropathy by modulating oxidative stress and improving renal function.

Our study aimed to further validate the potential of nano-formulated hesperidin in improving insulin sensitivity and glucose metabolism, potentially mediated by its effects on the PI3K signaling pathway. To accomplish this, we conducted gene expression analysis by RT-PCR on kidney tissues to examine the molecular effects of nano-formulated hesperidin compared to diabetic-induced, control, and standard drug groups. Our results revealed that the expression levels of key genes involved in the PI3K signaling pathway, including mTOR, PI3K, Glut4, and AS160, were upregulated in the diabetic-induced kidney tissues. Notably, the group treated with nano-formulated hesperidin exhibited reduced expression levels of these genes, as depicted in Fig. 3. These findings strongly suggest that nano-formulated hesperidin treatment modulates the PI3K/mTOR signaling pathways in kidney tissues. This evidence highlights the potential of nano-formulated hesperidin as a therapeutic agent for regulating insulin sensitivity and glucose metabolism, possibly through its modulation of the PI3K signaling pathway.

Continuing our study, we conducted protein expression analysis by western blot on kidney tissues to investigate the molecular effects of nano-formulated hesperidin in comparison to diabetic-induced, control, and standard drug groups. Our findings revealed upregulated expression levels of key genes involved in the PI3K signaling pathway, including mTOR, PI3K, Glut4, and AS160, in the diabetic-induced kidney tissues. However, the group treated with nano-formulated hesperidin exhibited reduced expression levels of these genes, as illustrated in Fig. 4. These results strongly suggest that nano-formulated hesperidin treatment modulates the PI3K/mTOR signaling pathways in kidney tissues. This evidence underscores the potential of nano-formulated hesperidin as a therapeutic agent for regulating insulin sensitivity and glucose metabolism, likely through its modulation of the PI3K signaling pathway. To further understand the potential regulatory role of hesperidin on the PI3K signaling pathway, molecular docking studies were conducted. The aim was to evaluate the interaction between hesperidin and key proteins involved in the PI3K pathway, namely mTOR, GLUT4, and AS160. The results indicated binding energies between hesperidin and these proteins, with mTOR displaying the highest binding energy at -10.4 kcal/mol, followed by GLUT4 (-11.2 kcal/mol) and AS160 (-8.6 kcal/mol), as illustrated in Figure 5. This suggests a strong potential for interaction between hesperidin and these proteins. These findings imply that hesperidin may directly interact with mTOR, GLUT4, and AS160, potentially modulating their activity. This indicates that hesperidin may indeed interfere with the PI3K/mTOR signaling pathway, thereby impacting diabetes.

This study evaluates nano-formulated hesperidin as a potential therapeutic for diabetic nephropathy in a rat model. By investigating molecular mechanisms, including PI3K/mTOR pathway modulation, it demonstrates reduced kidney function markers and downregulation of PI3K and mTOR proteins and transcripts with hesperidin treatment. Molecular docking confirms hesperidin's strong affinity for PI3K/mTOR targets. This comprehensive approach underscores nano-formulated hesperidin's promise in mitigating diabetic nephropathy, suggesting targeted treatment avenues. Overall, reductions in markers, protein expression, and gene transcription highlight hesperidin's potential in combating diabetic nephropathy, offering novel therapeutic prospects.

Conclusion

This research highlights a comprehensive approach involving biochemical, molecular, and computational analyses in a rat model, the study elucidated the intricate molecular mechanisms

underlying hesperidin's renoprotective effects, particularly through modulation of the PI3K/mTOR signaling pathway. The findings demonstrate that nano-formulated hesperidin effectively reduced kidney function markers, including urea and creatinine levels, indicating its potential to preserve renal function in diabetic nephropathy. Furthermore, downregulation of PI3K and mTOR protein expression, along with decreased transcript levels observed in kidney samples treated with nano-formulated hesperidin, provides molecular insights into its therapeutic influence on diabetic nephropathy. Overall, the study underscores the promising therapeutic efficacy of nano-formulated hesperidin in mitigating the deleterious effects of diabetic nephropathy. These findings pave the way for further research and development of nano-hesperidin-based interventions, offering new avenues for targeted and effective treatment of diabetic nephropathy, a chronic and debilitating complication of diabetes mellitus.

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Tables

Table 1. RT-PCR primers

Sl. No	Name	Sequence (5' - 3')	Reference(s)
1	mTOR-F	GGTGGACGAGCTCTTTGTCA	[32]
	mTOR -R	AGGAGCCCTAACACTCGGAT	
2	PI3K-F	AACACAGAAGACCAATACTC	[33]
	PI3K -R	TTCGCCATCTACCACTAC	
3	GLUT4-F	GAGCCTGAATGCTAATGGAG	[34]
	GLUT4-R	GAGAGAGAGCGTCCAATGTC3	
4	AS160-F	CCTAGCGCAGCCAGGTG	[35]
	AS160-R	TCCTGCGATCCAAGCAAGAC	
5	β-actin-F	CACCCGCGAGTACAACCTT	[33]
	β-actin-R	CCCATACCCACCATCACACC	

Table 2: Biochemical analysis of renal in Diabetic rats

Grouping	Control	Type 2 Diabetic Mellitus	Type 2 Diabetic Mellitus + NF-HSP	Type 2 Diabetic Mellitus + GBN	Control + NF-HSP
Body weight (b. wt.)	410.81±30	238.5±26.01	305.2±15	274.96±11	395±5
Urine volume (ml/hr)	20±1.57	151±2.8	123±3.2	135±3.1	21±3.7
Glycemia (mmol/l)	10.8±0.1	25±2.8	26.35±1.2	20.1±1.6	10.3±1.9
Albuminuria (mg/24h)	0.91±0.17	30.2±3.8	20.85±1.7	25.63±3.1	0.85±1.9
Blood glucose (mg/dl)	85.3±10	501.41±35	221.45±31	287.45±4.8	89.34±5.3

Table 3: Molecular docking analysis

Compound	Proteins	Binding energy (Kcal/mol)	No. of H bonds	Amino acid residues
Hesperidin	mTOR	-10.4	6	SER1176, CYS1174, SER1218, SER1173, PHE1309, PHE1031
	GLUT4	-11.2	4	GLY400, GLN298, ASN304, TRP428
	AS160	-8.6	4	ALA1094, THR977, MET1157

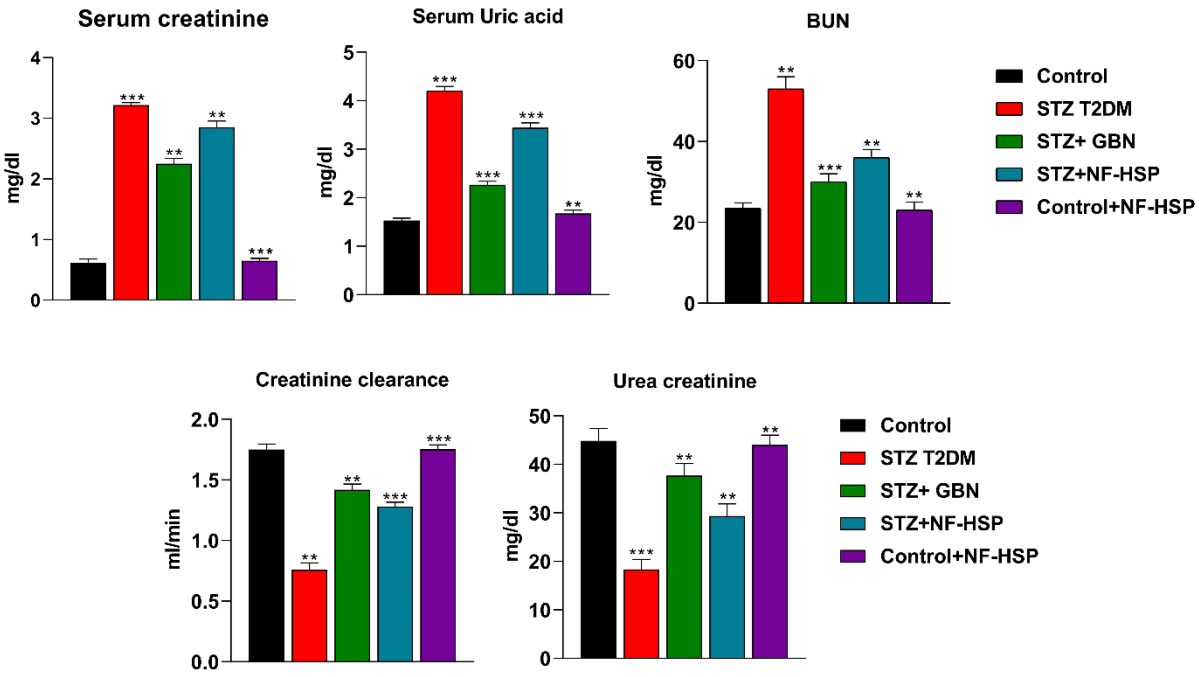

Fig. 1. Effect of nano-formulated hesperidin in renal functional markers (Seum creatinine, Serum uric acid, BUN, Creatinine clearance, and Urea creatinine) compared with control, STZ T2DM, STZ+GBN, and control+NF-HSP from kidney tissues. Statistically significant, **p<0.05 and ***p<0.001.

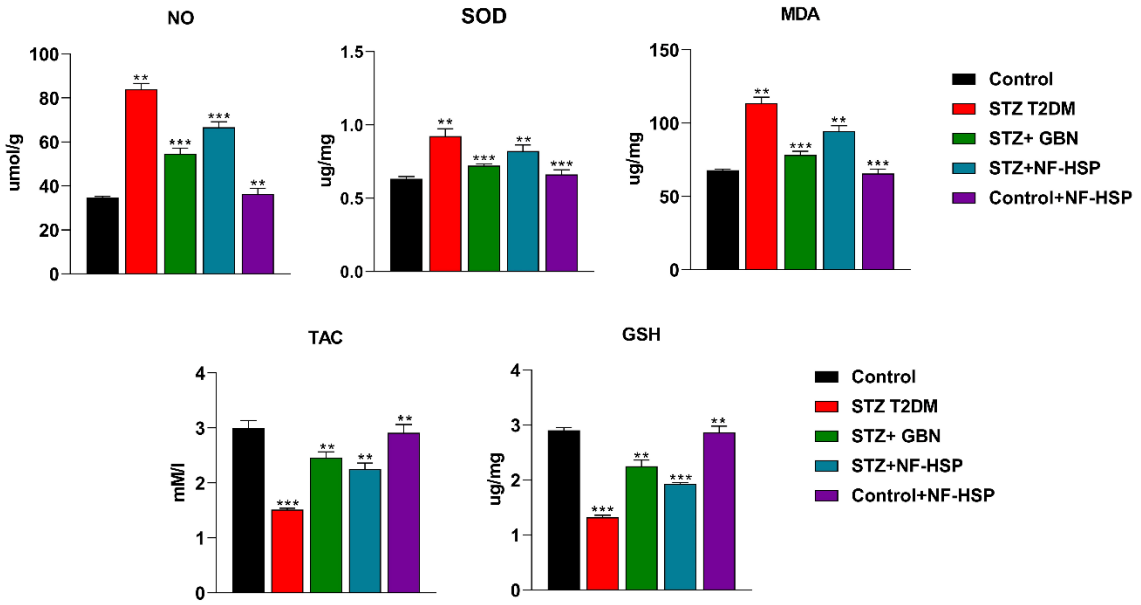

Fig. 2. Effect of nano-formulated hesperidin in anti-oxidant enzyme activity (NO, SOD, MDA, TAC, and GSH) compared with control, STZ T2DM, STZ+GBN, and control+NF-HSP from kidney tissues. Statistically significant, ***p*<0.05 and ****p*<0.001.

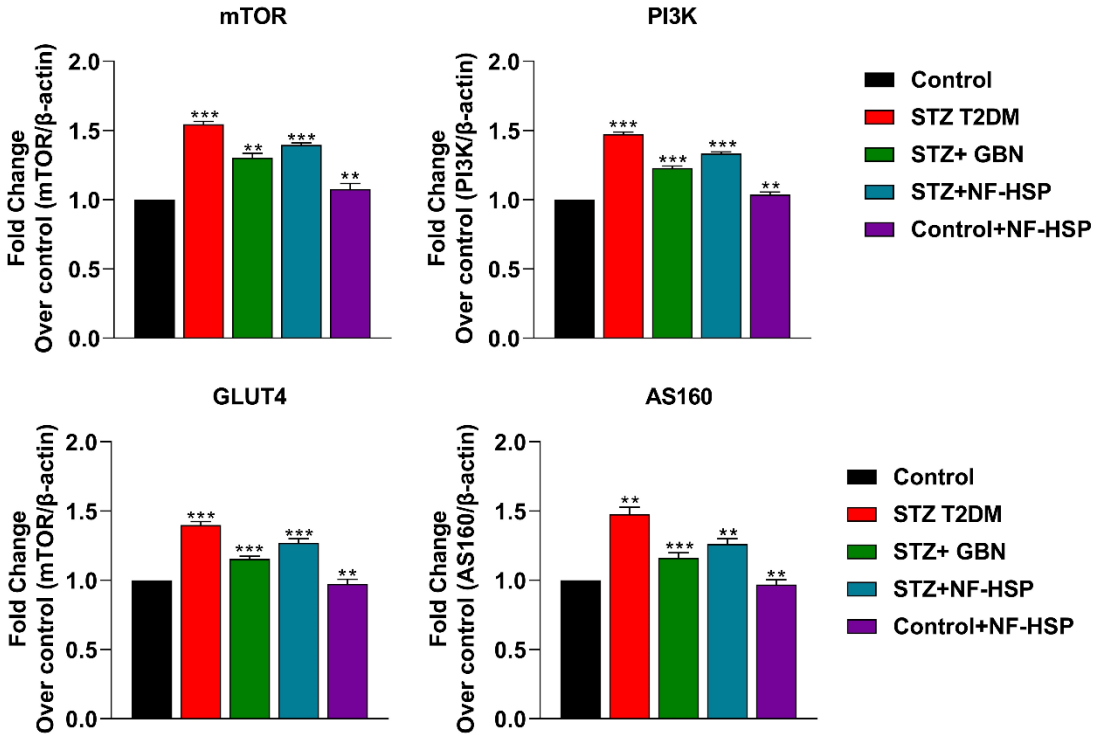

Fig. 3. Effect of Nano-formulated hesperidin in gene expression levels were measured by RT-PCR for mTOR, PI3K, Glut4, and AS160 compared with control, STZ T2DM, STZ+GBN, and control+NF-HSP from kidney tissues. Statistically significant, ***p*<0.05 and ****p*<0.001.

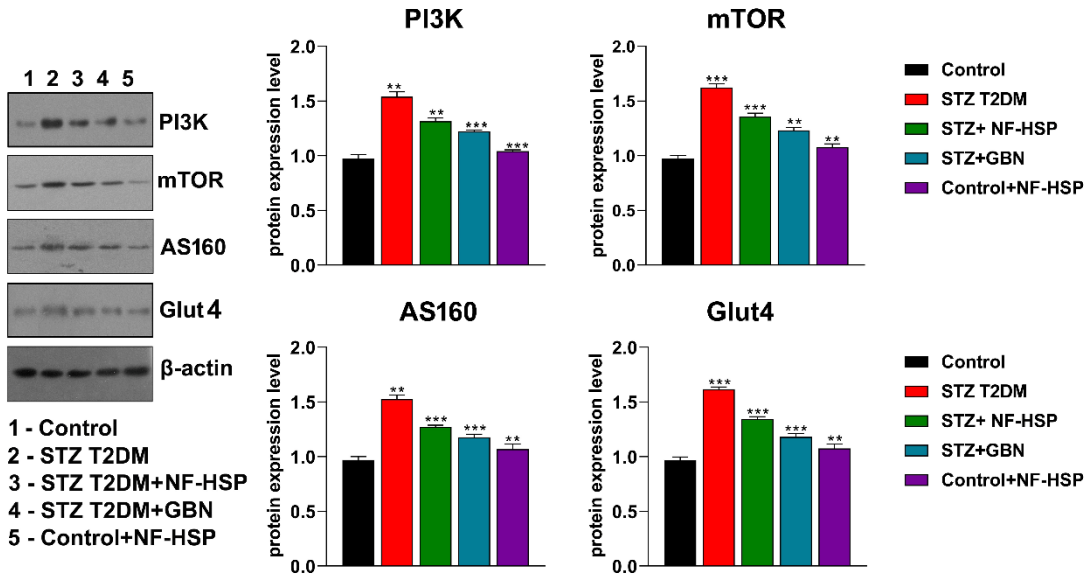

Fig. 4. Effect of nano-formulated hesperidin in protein expression levels were measured by RT-PCR for PI3K, mTOR, AS160, and Glut4 compared with control, STZ T2DM, STZ+GBN, and control+NF-HSP from kidney tissues. Statistically significant, ***p*<0.05 and ****p*<0.001.

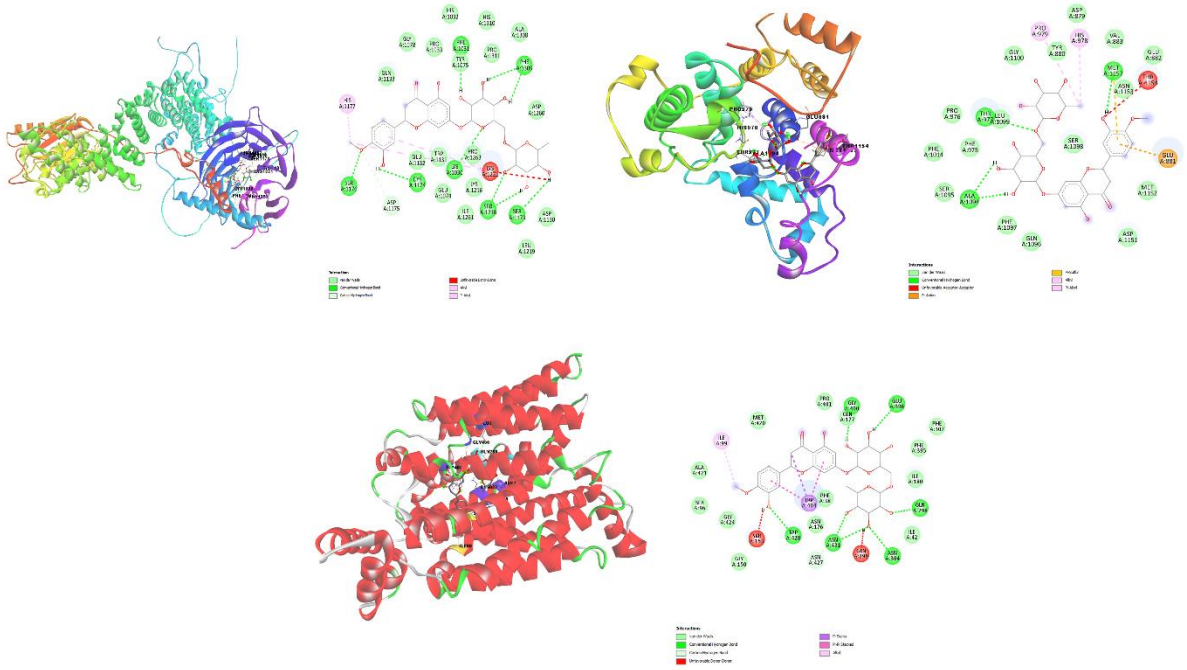

Fig. 5. Molecular docking result shows binding association by interaction of hesperidin against mTOR, AS160, and Glut4. #d and 2D illustration of binding complexes were visualized using Biovia discover studio.