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ABOUT COVER

Peer Review of World Journal of Diabetes, Dimiter Avtanski, PhD, Director, Endocrine Research Laboratory, Friedman Diabetes Institute, Lenox Hill Hospital, New York, NY 10022, United States. davtanski@northwell.edu

AIMS AND SCOPE

The primary aim of World Journal of Diabetes (WJD, World J Diabetes) is to provide scholars and readers from various fields of diabetes with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WID mainly publishes articles reporting research results and findings obtained in the field of diabetes and covering a wide range of topics including risk factors for diabetes, diabetes complications, experimental diabetes mellitus, type 1 diabetes mellitus, type 2 diabetes mellitus, gestational diabetes, diabetic angiopathies, diabetic cardiomyopathies, diabetic coma, diabetic ketoacidosis, diabetic nephropathies, diabetic neuropathies, Donohue syndrome, fetal macrosomia, and prediabetic state.

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The WID is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The 2024 Edition of Journal Citation Reports® cites the 2023 journal impact factor (JIF) for WJD as 4.2; JIF without journal self cites: 4.1; 5-year JIF: 4.2; JIF Rank: 40/186 in endocrinology and metabolism; JIF Quartile: Q1; and 5year JIF Quartile: Q2.

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Basic Study

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ORIGINAL ARTICLE

Tiliroside protects against diabetic nephropathy in streptozotocininduced diabetes rats by attenuating oxidative stress and inflammation

Yan Shang, Cai-Yun Yan, Hui Li, Na Liu, Hui-Feng Zhang

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Abstract

BACKGROUND

Diabetic nephropathy (DN), affecting half of diabetic patients and contributing significantly to end-stage kidney disease, poses a substantial medical challenge requiring dialysis or transplantation. The nuanced onset and clinical progression of kidney disease in diabetes involve consistent renal function decline and persistent albuminuria.

AIM

To investigate Tiliroside's (Til) protective effect against diabetic nephropathy (DN) in rats under diabetic conditions.

METHODS

Five groups of six rats each were included in this study: Rats treated with DMSO by intraperitoneal injection as controls, those treated with STZ by intraperitoneal injection, those treated with STZ + Til (25 mg/kg body weight [bwt]) or Til (50 mg/kg bwt), and those treated with anti-diabetic medication glibenclamide (600 μ g/kg bwt). Biochemical markers, fasting blood glucose, food intake, kidney weight, antioxidant enzymes, inflammatory and fibrotic markers, and renal injury were monitored in different groups. Molecular docking analysis was performed to identify the interactions between Til and its targeted biomarkers.

RESULTS

Til significantly reduced biochemical markers, fasting blood glucose, food intake, and kidney weight and elevated antioxidant enzymes in diabetic rats. It also mitigated inflammatory and fibrotic markers, lessened renal injury, and displayed inhibitory potential against crucial markers associated with DN as demonstrated



by molecular docking analysis.

CONCLUSION

These findings suggest Til's potential as a therapeutic agent for DN treatment, highlighting its promise for future drug development.

Key Words: Tiliroside; Diabetic nephropathy; Antioxidant; Diabetes mellitus; Oxidative stress; Inflammation

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Core Tip: Tiliroside (Til) demonstrates potent protective effects against diabetic nephropathy (DN) in rats, alongside glibenclamide. Through attenuating oxidative stress, inflammation, and fibrosis, Til treatment significantly improves renal function and reduces biochemical markers associated with DN. Molecular docking analysis reveals its potential inhibition of key markers linked to the disease. These findings underscore Til's promising role as a therapeutic agent for DN, suggesting avenues for future drug development.

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INTRODUCTION

Diabetes mellitus (DM), a prevalent and potentially fatal medical condition, has been linked to various cardiovascular disorders, including stroke, peripheral arterial disease, coronary heart disease, retinopathy, kidney disorder, and peripheral neuropathy[1]. The incidence of diabetes has elevated significantly over the world in recent decades as a consequence of alterations in lifestyle and socioeconomic development[2]. A serious chronic consequence of DM types 1 and 2 is diabetic nephropathy (DN), the leading global cause of dialysis, renal failure, and transplantation[3]. The prevalence of DN peaks after 10 to 20 years of diabetes and is a chronic kidney disease that gradually progresses[4]. DN, a metabolic illness, is the primary cause of end-stage renal failure, characterized by renal hyperfiltration, early microalbuminuria, and increased permeability to protein, urea, and macromolecules[5]. Modifiable risk factors include glycemic control, dyslipidemia, hypertension, age, ethnicity, and genetic profile, while inevitable factors include family history and men's higher risk[6].

Reactive oxygen species (ROS) are produced in higher concentrations as a result of oxidative stress induced by hyperglycemia[7]. ROS cause cell membrane destruction, deactivation of anti-oxidant enzymes, and modifications in the expression of endogenous anti-oxidant genes, and contribute to the development of DN[8]. ROS trigger signal transduction cascades, causing profibrotic molecules like collagen IV, fibronectin, and lamin expression, and promoting extracellular matrix build-up and inflammatory gene expression. It also promotes tissue fibrosis and cell proliferation[9]. New research shows that angiotensin receptor blockers (ARBs) and sodium-glucose cotransporter-2 inhibitors are effective in treating DN, halving renal disease risk by 45%, but adverse effects limit their clinical use[10].

Since hundreds of years ago, several medicinal plants have been employed often in the management of diabetes and its complications. For potential in vitro inhibitory actions on advanced glycation end-products (AGEs), various plant extracts have recently undergone screening[11]. Plant flavonoids exhibit anti-allergic, anti-inflammatory, hepatoprotective, anticarcinogenic, antiviral, and anti-thrombotic properties, primarily due to their antioxidant activity, which involves chelation, elimination, and repression of ROS-producing enzymes[12]. A glycosidic flavonoid called Tiliroside (Til) is present in several food and medicine sources, including linden, rose hips, and strawberries[13]. There have been no reports of Til toxicity against non-cancerous cells, and it has been shown to have antioxidant, antiobesity, antidiabetic, and other properties[14].

Til, a flavonoid glycoside, has antioxidant properties and potential benefits in diabetes treatment, particularly DN. It modulates signaling pathways related to DN, including inflammation and apoptosis[15]. Til's antioxidant properties mitigate oxidative damage caused by hyperglycemia, which is crucial for DN pathogenesis. It also influences inflammatory pathways, potentially reducing inflammatory responses in DN[16]. Additionally, it reduces apoptosis in renal cells, preserving renal function. Further research is needed to understand its molecular mechanisms and potential clinical applications. Til's antioxidant effects also influence various inflammatory pathways, which are critical factors in DN[17]. By downregulating pro-inflammatory cytokines and pathways involved in inflammation, Til could potentially reduce inflammatory responses that exacerbate DN. Additionally, Til may reduce apoptosis in renal cells, which is a significant contributor to renal cell death in DN[18].

The primary goal of the current study was to evaluate Til's effectiveness against streptozotocin (STZ)-provoked diabetes in SD rats by examining the biochemical and histological markers. The impact of Til on levels of fasting blood glucose (FBG), food intake, body weight (BW), water intake, insulin, kidney weight, antioxidants and pro-inflammatory



markers, biochemical markers, and fibrotic markers levels in untreated and treated rats was examined.

MATERIALS AND METHODS

Materials

Glibenclamide (GB), Til, and STZ were purchased from Sigma-Aldrich in the United States. All of the other necessary chemicals and reagents were of high analytical category.

Animal housing and dietary treatments

After receiving approval from the Institutional Review Board, mature healthy Wistar rats weighing 180-200 g were purchased from the Animal Facility. In a fully sanitary laboratory, the rat adaptation was carried out by keeping the temperature at 25 °C, the relative humidity at 55 °C for a duration of 1 wk, and the cycle of daylight and darkness for 12 h. Following adaptation, rats were fed regular rat food, and freshwater was always supplied. All experimental techniques utilized in the current study were approved by the local ethics committee, and the rats were handled with the utmost care.

Diabetes induction

Using the medication STZ, diabetes was triggered in the experimental animals. STZ (60 mg/kg) was intraperitoneally provided to the animals in a 0.1 M citrate buffer at pH 4.4. The blood glucose level was measured to verify the onset of diabetes after 7 d of STZ therapy. In the current experimental investigation, rats having a blood glucose value of more than 11 mmol/L were used.

Experimental design and sampling

Five groups of six rats each were used in the study. Groups were randomly split. The first group of rats (control group) received an intraperitoneal injection of about 0.5% of DMSO. The second group of rats received an intraperitoneal injection of the medication STZ at a dose of 60 mg/kg. The third group received Til (25 mg/kg body weight [bwt]) after being given STZ. The fourth group received Til (50 mg/kg bwt) after being given STZ. The fifth group received the antidiabetic medication GB (600 µg/kg bwt) after being given STZ. GB and Til, dissolved in 0.5% DMSO, were given orally once daily in the morning for 60 d. Following the conclusion of the course of therapy, the rats were sacrificed, their blood was obtained for biochemical analysis, and their kidney, liver, and pancreas tissues were obtained for histological research.

Estimation of FBG and BW of animals

The BW of the experimental animals was recorded on the 0th and final day of the experiment and compared between all experimental groups. On the 0th, 30th, and 60th days following the administration of medication, fasting blood samples were taken. Before measuring the FBG, the rats had a fast for an entire night. Precisely drawn orbital sinus blood samples were used to estimate glucose levels utilizing the glucose assessment kit.

Analysis of food intake, homeostasis model assessment of insulin resistance, kidney weight, water intake, and insulin levels of STZ-provoked animals

Regular measurements of food and water consumption were taken for each group of rats during the course of the study. All of the rats in the group were killed under anesthetic circumstances (24 mg/kg BW of ketamine intramuscularly). Blood was extracted into collection tubes both with and without anticoagulants. Immediately after being separated, the kidney and liver were weighed and thoroughly cleansed in ice-cold saline to remove any blood. After that, 0.1 M Tris-HCl buffer was used to yield a 10% homogenate, which was then centrifuged at 1000 g for a duration of 10 min. Tests on biochemical parameters were conducted using the isolated supernatants. An ELISA kit that is available commercially was used to measure the insulin levels in the test animals. A microplate ELISA reader was used to measure the final absorbance of the standard and sample at 450 nm just after the stop solution was added. The homeostasis model assessment of insulin resistance (HOMA-IR) was employed to determine the level of plasma insulin resistance in the experimental animals. The subsequent formula was used to compute HOMA-IR using fasting glucose and insulin values: HOMA-IR = fasting glucose $(mmol/L) \times fasting insulin (uIU/mL)/22.5$.

Estimation of blood urea nitrogen, serum creatinine, uric acid, N-acetyl glucosamine, and urinary microalbumin levels in STZ-provoked animals

To evaluate the renal function, serum was centrifuged at 3600 g for 10 min. On an automated biochemical analyzer, the blood urea nitrogen (BUN) and serum creatinine (SCr) levels were measured. The levels of N-acetyl glucosamine (NAG), uric acid, and urinary microalbumin (U-mAlb) were measured from the collected urine samples by utilizing respective commercially available kits.

Estimation of anti-oxidant and oxidative stress biomarker levels in STZ-provoked animals

The levels of anti-oxidant biomarkers including glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione (GSH) were estimated calorimetrically utilizing commercially available kits by following the protocols



provided by the manufacturer. ELISA kits were used following the instructions provided by the manufacturer to determine the levels of plasma AGEs. The production of intracellular ROS in rat kidney homogenate was fluorometrically measured by oxidizing the nonfluorescent label 2,7-dichloro fluorescein diacetate (DCFDA) to the fluorescent products dichloro fluorescein (DCF). Briefly, 5 μ mol/L DCF-DA was added to 30 μ L of kidney homogenate in phosphate-buffered saline, and the mixture was then left to sit at room temperature for 30 min. Following that, the mean fluorescence intensity was directly measured at 485 nm and 535 nm for the excitation and emission wavelengths, respectively.

Estimation of pro-inflammatory markers in STZ-provoked animals

The levels of pro-inflammatory markers including interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β) in each group were measured using commercial ELISA kits that were acquired from the market. The experiment followed the instructions given by the manufacturer. The experiment was done in triplicate, and the absorbance at 450 nm was determined. The standard curve with established standard concentrations was used to compute the final results.

Estimation of fibronectin and transforming growth factor-beta 1 levels in STZ-provoked animals

The assessment of fibronectin and transforming growth factor-beta 1 (TGF- β 1) levels in the renal tissue was carried out utilizing ELISA kits. The renal cortex was centrifuged at a speed of 9000 rpm for a duration of 20 min at 4 °C after being instantly homogenized in ice-cold phosphate buffer saline with 0.05% Tween 20. The supernatants were then used to estimate the concentrations of fibronectin and TGF- β 1 using ELISA kits that were commercially available.

Histopathological studies

All five investigation groups-Control, diabetic provoked, diabetic provoked and Til-treated (25 mg/kg and 50 mg/kg), and diabetic-provoked & GB-treated had their renal tissues subjected to histopathological analysis. Initial formalin (10%) treatment of the tissues was carried out for 24 h. The tissue was subsequently sliced into 3-5 μ m thick sections with a rotary microtome, embedded in paraffin, and stained with hematoxylin and eosin (HE). The stained slides were subsequently observed using an Olympus light microscope.

Statistical analysis

All statistical analyses were completed using GraphPad Prism version 6.01. The results are reported as the mean \pm SD. ANOVA and the Tukey *post hoc* test were used to check whether there were any differences between the groups. *P* values < 0.05 were deemed statistically significant.

Molecular modeling and docking approach

Homology modeling and its validation: Due to the lack of three-dimensional (3D) structure for TGF- β 1 (UniProt ID: Q3UNK5) in the PDB, the TGF β 1 structure was modeled using the alpha fold2 method integrated with the SWISS model. The Ramachandran plot (RP) is a highly valuable tool for validating protein structures. It displays the mapping of φ/ψ torsion angles of the polypeptide backbone in pairs against the predicted or "allowed" values. In this context, the modeled 3D structure of TGF- β 1 was verified using RP to identify an amino acid corrosive in a protein structure by the use of Verify3D and ERRAT through SAVES server (https://saves.mbi.ucla.edu/; Accessed on 17 November 2023), which demonstrated specific details regarding the stability, consistency, and dependability of the protein's tertiary structure.

Active site prediction: The active sites for the described biomarkers, such as fibronectin and TGF- β 1, were predicted using PrankWeb, which provides an interface to P2Rank, a template-free machine learning method to detect the ligand binding sites (http://prankweb.cz/; accessed on 18 November 2023)[19].

Preparation of protein structure and ligand: The 3D structure of the described rat renal fibrotic markers such as fibronectin (PDB ID: 1MFN) and TGF- β 1 (UniProt ID: Q3UNK5) and pro-inflammatory biomarkers such as IL-6 (PDB ID: 2 L3Y), TNF- α (PDB ID: 2TNF), and IL-1 β (PDB ID: 4G6M) were retrieved from the Protein Data Bank and UniProt[20-23]. The water molecules and associated ligands were removed for the retrieved biomarkers using the Discovery Studio Visualizer v19.1.0.18287 (www.accelerys.com). The ligand Til was downloaded from the PubChem compound database [24] and saved in the SDF file format with the provided atomic coordinates and converted into PDB format using Discovery Studio Visualizer v19.1.0.18287 (www.accelerys.com).

Molecular docking and visualization

After preparing the chemical compound Til as a ligand, and fibronectin (PDB ID: 1MFN), TGF-β1 (UniProt ID: Q3UNK5), IL-6 (PDB ID: 2 L3Y), TNF-α (PDB ID: 2TNF), and IL-1β (PDB ID: 4G6M) as targets, we used PyRx software to implement Autodock Vina for the molecular docking process. The docking propensity of Til and the interfaces between Til and the target residues are examined. The prepared targets and ligand were saved in PDBQT format. The size was assigned to the grid box properties as fibronectin (size_x = 23.44 Å, y = 20.53 Å, and size_z = 23.61 Å), TGF-β1 (size_x = 27.67 Å, y = 24.01 Å, and size_z = 23.00 Å), IL-6 (size_x = 28.37 Å, y = 25.22 Å, and size_z = 34.11 Å), TNF-α (size_x = 18.36 Å, y = 19.34 Å, and size_z = 18.64 Å), and IL-1β (size_x = 55.68 Å, y = 52.27 Å, and size_z = 51.22 Å). The PyRx virtual screening tool identified the interacted active site residues of the targeted biomarkers inhibited by Til. The interactions between Til and its targeted biomarkers were visualized in 3D by importing the docked data into PyMol, Lig-Plot+, and Discovery Studio Visualizer v19.1.0.1828 [Dassault Systèmes BIOVIA, Rue Marcel Dassault, Vélizy-Villacoublay-78140, France, www. accelerys.com (accessed on 20 November 2023)]. Additionally, the 2D diagram was obtained for the interacted ligand and

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Figure 1 Effect of Tiliroside on fasting blood glucose level in experimental rats. A-C: Fasting blood glucose (FBG) at day 7 (A), day 30 (B), and day 60 (C). Data are given as the mean \pm SD of three individual values. All results were statistically assessed by one-way ANOVA and Tukey's *post hoc* test. ^a*P* < 0.05 *vs* Control; ^b*P* < 0.01 *vs* STZ. FBG: Fasting blood glucose; GB: Glibenclamide; TIL: Tiliroside; STZ: Streptozotocin.



Figure 2 Effect of Tiliroside on initial and final body weight of experimental rats. A: Initial body weight; B: Final body weight. Data are given as the mean ± SD of three individual values. All results were statistically assessed by one-way ANOVA and Tukey's *post hoc* test. ^a*P* < 0.05 *vs* Control; ^b*P* < 0.01 *vs* STZ. GB: Glibenclamide; TIL: Tiliroside; STZ: Streptozotocin.

targets using LigPlot⁺. A molecular docking study was carried out using Autodock Vina by utilizing PyRx which offers an integrated scoring algorithm.

RESULTS

Impact of Til on FBG levels in experimental rats

Figure 1 depicts the impact of Til on the FBG level in the experimental animals on the 7th, 30th, and 60th days. On the 7th day, the level of blood glucose was almost the same for all the experimental groups except the control group. On the 30th day, the blood glucose level was decreased in all groups in comparison to the STZ-treated control animals (group II). Whereas on the 60th day, there was a significant decrease in the blood glucose level in animals treated with Til in a dose-based manner (groups III and IV). At the end of the study, there was no considerable change in the FBG level between the Til -treated and GB-treated animals.

Impact of Til on body weight of experimental rats

Figure 2 depicts the effect of Til on the BW in STZ-treated rats. On the 0th day, the BW of the experimental animals in all the groups was the same. Group II (STZ-treated) animals showed a reduction in their BW in comparison to the STZ-induced animals treated with Til extract in a dose-dependent manner (group III and group IV). Til extract almost



maintained the animal BW in group III and group IV.

Impact of Til on food consumption, HOMA-IR, kidney weight, water intake, and insulin levels in experimental animals

In the DR animals, the effects of Til medication on many parameters, including food intake, kidney weight, HOMA-IR, water consumption, and insulin levels, were investigated. The average food intake, HOMA-IR, kidney weight, and water consumption levels of the STZ-induced DR animals were shown to be higher when compared to those of control animals. In STZ-induced mice, Til treatment dramatically decreased food intake, HOMA-IR, kidney weight, and water consumption. Additionally, compared to rats exposed to STZ, Til treatment elevated the insulin level in animals (Figure 3). The effects of GB treatment were comparable to those of Til.





Impact of Til on renal biochemical markers in experimental rats

Figure 4 illustrates the effect of Til on the serum levels of BUN, SCr, uric acid, NAG, ROS, and microalbumin in STZtreated rats. The findings revealed that 24 h after receiving STZ, the rats had acute nephrotoxicity as evidenced by a substantial rise in BUN, uric acid, SCr, NAG, and microalbumin levels. When rats with nephrotoxicity induced by STZ were treated with Til (group III and group IV), the levels of BUN, SCr, NAG, uric acid, and microalbumin were significantly decreased in a dose-dependent manner when compared with those of the STZ-treated animals (group II). The effects of GB treatment were comparable to those of Til.

Impact of Til on the antioxidant and oxidative stress markers in experimental rats

The effect of Til treatment on the levels of different antioxidants in untreated and treated rats was evaluated. Figure 5 shows that STZ-stimulated animals had considerably decreased levels of antioxidants including SOD, GSH, and GPx in their retinal tissues when compared to control animals. It's noteworthy to note that Til treatment dramatically increased the SOD, GSH, and GPx in the STZ-induced rats. The level of ROS and plasma AGE level were elevated in the STZ-



Figure 4 Effect of Tiliroside on renal biochemical markers in experimental rats. A: Blood urea nitrogen (mmoL/L); B: Serum creatinine (μ moL/L); C: Uric acid (μ moL/L); D: N-acetyl glucosamine; E: U-mAlb (urinary microalbumin). All results were statistically assessed by one-way ANOVA and Tukey's *post hoc* test. ^a*P* < 0.05 *vs* Control; ^b*P* < 0.01 *vs* STZ. GB: Glibenclamide; TIL: Tiliroside; STZ: Streptozotocin; BUN: Blood urea nitrogen; SCr: Serum creatinine; NAG: N-acetyl glucosamine.

provoked animals in comparison to control rats. On treatment with Til, the levels of ROS and plasma AGEs were reduced. Administration of Til increased antioxidant state in the STZ-provoked DR rats.

Impact of Til on pro-inflammatory markers in experimental rats

The levels of IL-6, TNF- α , and IL-1 β were measured in the retinal tissues of untreated and treated rats and are shown in Figure 6. The levels of IL-6, TNF- α , and IL-1 β in the retinal tissues were significantly increased in the STZ-stimulated rats as compared to the control animals. When Til was administered as a supplement, the levels of IL-6, TNF- α , and IL-1 β expression in the STZ rats, however, significantly decreased (Figure 6). The expression of these markers in the retinal tissue of STZ-provoked animals was likewise decreased by GB treatment.

The 3D modeled structure of transforming growth factor-beta 1 (UniProt ID: Q3UNK5) is a crucial validation tool for 3D protein structures, assessing the quality of backbone conformation. It visualizes allowed and disallowed regions for dihedral angles, indicating rotation around bonds. Interpreting the Ramachandran Plot helps identify allowed and disallowed regions, red flags, and outliers (Figure 7).

Additionally, the molecular docking analysis was performed to identify the crucial interactions of Til with the described pro-inflammatory and renal fibrotic markers. In this context, we focused on the task of predicting active sites using the target 3D structures which include IL-6, TNF- α , IL-1 β , fibronectin, and TGF- β 1 (Figure 8). The predicted active site residues of the targets were finalized based on the first ranking method which uses the machine learning approach P2Rank (Table 1). The molecular docking analysis was performed between Til and the predicted active site of the targeted biomarkers. The resulting molecular docking analysis confirmed that Til interacted with the residues of IL-6 such as SER93 and ILE92 *via* hydrogen bond and pi-sigma bond formed with VAL136. Additionally, the pi-cation, pi-alkyl, and pi-pi T-shaped bonds formed with the residues such as CYs78, CYS88, and HIS 140. It was also surrounded by hydrophobic residues such as TYR83, ASN84, GLN85, GLU86, ILE87, LEU89, LEU90, LYS91, ASN133, PHE132, LEU139, and ILE142 with a binding affinity of -8.4 kcal/mol and root mean square distance (RMSD) of 2.652Å (Figure 8C and D; Table 2). Likely, Til interacted with TNF- α residues such as SER99, LYS112, and TYR115 *via* hydrogen bond with a

Table 1 Predicted active sites for fibronectin, transforming growth factor-β1, interleukin-6, and tumor necrosis factor-α with the rank

No.	Biomarker name	Targets (PDB ID)	Rank	Score	Probability	Predicted active sites
1	Fibronectin	1MFN	1	2.74	0.084	HIS36, HIS37, ALA38, SER41, ARG46, ASN61, LEU62, ASN63, TYR68
2	TGF-β1	Q3UNK5	1	9.11	0.534	ARG277, THR282, ASN283, ASN292, CYS293, CYS294, TRP330, ASP333, GLN359, SER386, CYS387, CYS389, PRO99
3	IL-6	2 L3Y	1	10.37	0.597	ILE129, PHE132, ASN133, GLU135, VAL136, ILE142, LEU184, THR187, ARG188, THR27, VAL30, GLY31, LEU89, LEU90, LYS91, ILE92, SER93, LEU96
4	TNF-α	2TNF	1	8.36	0.491	PRO100, PRO102, TRP114, GLU116, LYS98, SER99

P2Rank is a machine learning tool that predicts ligand-binding sites in proteins using structural features, ranking them based on their likelihood of being active sites. TGF-β1: Transforming growth factor-β1; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α.

Table 2 List of targets									
Target name	Target PDB/UniProt ID	Binding affinity (kcal/mol)	RMSD (Å)	Type of interacted bonds					
				Hydrogen bond	Hydrophobic residues	Other bonds			
Fibronectin	1MFN	-7.6	2.583	ARG44, ASN61	HIS37, HIS40, SER41, GLY43, PRO45, LEU62, ASN63, THR66, TYR68,	ALA38 ^{pa} , ARG46			
IL-6	2 L3Y	-8.4	2.652	ILE92, SER93(2)	LYS91, LEU89, ILE142, LEU90, ASN133, PHE132, LEU139, ILE87, GLU86, GLN85, TYR83, ASN84	VAL136 ^{psa} , CYC78 ^{ps} , CYC88 L ^{ps}			
TNF-α	2TNF	-5.4	1.816	SER99, TYR115, LYS112	GLY68, VAL97, PRO102, LYS103, PRO105, PRO106, TRP114, PRO113, GLU116,	LYS98 ^{pc, ch} , CYS69 ^{pa}			
IL-1β	4G6M	-7.5	2.062	TYR24, LYS77(2), LEU82(2)	GLU25, THR79, SER125, MET130, VAL132	LYS74 ^{pc} , PRO131 ^{pa} , PHE133 ^{pps (2)}			
TGF-β1	Q3UNK5	-9.1	2.125	GLU96, GLU100, ARG277, LEU280, THR282, ASN283, CYS293, GLN359	PRO95, PRO99, SER274, ARG275, ARG278, ALA279, ASN292, CYS326, TRP330, SER386, CYS389,	GLU98 ^{pc} , GLU100 ^{pc} , ARG277 ^{ps, pa} , ASN ^{283ch} , HIS276 ^{ch} , ASP333 ^{ch}			

TGF-β1: Transforming growth factor-β1; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β.

binding affinity of -5.4 kcal/mol and RMSD of 1.816Å. Also, the pi-cation and pi-alkyl bonds formed with the residues CYS69 and LYS98. Additionally, the hydrophobic residues such as VAL97, GLU116, TRP114, GLY68, PRO113, PRO102, THR105, LYS103, PRO106, and LYS103 were surrounded with the Til-IL-6 complex (Figure 8E and F; Table 2). Also, Til interacted via hydrogen bonds with the residues of IL-1beta which include TYR24, LYS77(2), and LEU82(2). Likely, the pication, pi-alkyl, and pi-pi sigma bonds are formed with the residues LYS74, PRO131, and PHE13(2). Also, the hydrophobic residues were surrounded by the Til-IL-1beta complex with a binding affinity of -7.5 kcal/mol and RMSD of 2.062Å (Figure 8G and H; Table 2).

Impact of Til on renal fibrotic markers in experimental rats

The effect of Til treatment on fibrotic markers including TGF-β1 and fibronectin in untreated and treated rats was evaluated, as depicted in Figure 9. The levels of fibronectin and TGF- β 1 were significantly increased in the STZstimulated rats as compared to the control animals. When Til was administered as a supplement, the levels of TGF-β1 and fibronectin in the STZ rats, however, considerably decreased. The effects of GB treatment were comparable to those of Til.

Due to the lack of TGF-β1 (Uniprot ID: Q3UNK5) structure, the Q3UNK5 structure was modeled, ranging between amino acid residues 1-390, using the TGF- β 1 from *Mustela putorious* with a sequence identity of 90.00% (Figure 10A). The modeled TGF-B1 3D structure showed 87.8% residues in the most favored regions and 11.30% in additional allowed regions, indicating that the model can be taken into further study (Figure 10B). The molecular docking analysis of fibronectin found that that Til formed two hydrogen bonds with the residues ARG44 and ASN61 and a pi-alkyl bond with ALA38. Also, the Til-fibronectin complex surrounded by hydrophobic residues such as HIS37, HIS40, SER41, GLY43, PRO45, LEU62, ASN63, THR66, and TYR68 with a binding affinity of -7.6 kcal/mol and RMSD of 2.583Å (Figure 8A and B; Table 2). Above all, Til interacted well with the TGF-β1 via hydrogen bonds such as GLU96, GLU100, ARG277, LEU280, THR282, ASN283, CYS293, and GLN359 (Figure 10C-E). Also, the pi-cation bond formed with GLU98 and GLU100. The pi-sulfur and pi-alkyl bonds formed with ARG277. Additionally, the carbon-hydrogen bond formed with ASN283, HIS276, and ASP333 with a binding affinity of -9.1 kcal/mol and RMSD of 2.125Å (Figure 8I and J; Table 2).

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Figure 5 Effect of Tiliroside on oxidative stress and antioxidant biomarkers in experimental rats. A: Superoxide dismutase; B: Glutathione; C: Glutathione peroxidase; D: Reactive oxygen species; E: Advanced glycation end products. All results were statistically assessed by one-way ANOVA and Tukey's *post hoc* test. ${}^{a}P < 0.05$ vs Control; ${}^{b}P < 0.01$ vs STZ. GPx: Glutathione peroxidase; SOD: Superoxide dismutase; GSH: Glutathione; GB: Glibenclamide; TIL: Tiliroside; STZ: Streptozotocin; ROS: Reactive oxygen species; AGEs: Advanced glycation end products.

The overall resulting binding affinity, RMSD, interacting residues, and the types of bonds formed between the targeted biomarkers and Til are listed in Table 2.

Impact of Til on renal histopathology of experimental rats

The renal tissues from animals treated with Til and GB as well as control animals underwent histological examination (Figure 11). Renal tissues stained with H&E showed that STZ caused glomeruli to contract and necrotize, renal tubular epithelial cells to be damaged, edema, and neutrophil infiltration. When Til (groups III and IV) and GB (group V) were given to diabetic rats, histological alterations such as edema, tubular and glomerular injury, and neutrophil penetrations in the renal tissues were all minimized.

DISCUSSION

In individuals with type 1 or type 2 DM, nephropathy continues to be a major source of morbidity and significant predictor of death. The development of proteinuria, which is usually accompanied by a gradual deterioration in renal function, is one of the characteristics of DN. From an epidemiological, pathophysiological, and clinical standpoint, the condition is typically accompanied by high blood pressure and poor glycemic control[25]. Numerous investigations on diabetes and its consequences frequently employ the STZ-induced mouse model of the disease[26]. Investigations dependent on the pathogenic components of DN have led to the development of many new therapy options, including intensive glycemic management, precise control of blood pressure, ideal renin-angiotensin-aldosterone system blockade with angiotensin-converting enzyme inhibitors/ARBs, nutritional and physical activity, and a large number of novel agents[27]. Nowadays, many medications are derived from plants since they offer therapeutic benefits and are often devoid of side effects. Several chemical substances found in plants that are utilized for the treatment of DN include





Figure 6 Effect of Tiliroside on inflammatory markers in experimental rats. A: Tumor necrosis factor- α ; B: Interleukin-6; C: Interleukin-1 β . All results were statistically assessed by one-way ANOVA and Tukey's *post hoc* test. ^a*P* < 0.05 *vs* Control; ^b*P* < 0.01 *vs* STZ. GB: Glibenclamide; TIL: Tiliroside; STZ: Streptozotocin; TNF- α : Tumor necrosis factor- α ; IL-6: Interleukin-6; IL-1 β : Interleukin-1 β .



Figure 7 Representation of 3D modeled structure and its validation using Ramachandran Plot. A: The 3D modeled structure of transforming growth factor-beta 1 (UniProt ID: Q3UNK5); B: Ramachandran Plot.

glycosides, alkaloids, terpenoids, and flavonoids[28]. The emphasis of the current study was to investigate the way that a naturally occurring substance called Til affected the progression of problems related to diabetes in STZ-stimulated SD rats. To compare Til's anti-diabetic activity, GB, a common medication, was used.

STZ, a pancreatic medication that kills the cells located in the islets of Langerhans, was administered to SD rats as part of this investigation. As a result, the amount of insulin secreted significantly decreased while blood glucose levels surged [29]. A significant increase in fasting blood sugar was observed after STZ treatment, and these findings are consistent with previous research[30]. In the current investigation, treatment with Til significantly reduced the level of fasting glucose in the STZ-provoked animals in a dose-based manner. This finding demonstrates the anti-diabetic properties of Til. Animals treated with STZ develop diabetic conditions and it is related to excessive weight loss due to hypoinsulinemia, increased muscle wasting, hyperglycemia, and loss of tissue proteins[31]. After receiving Til medication, the BW of STZ-diabetic rats dramatically increased, showing that the hyperglycemia-related deterioration of muscle tissue had

A

Fibronectin (PDB ID: 1MFN)







Ε



G

IL-Iβ (PDB ID: 4G6M)







D IL6 (PDB ID: 2L3Y)



F TNFα (PDB ID: 2TNF)



Η IL-Iβ (PDB ID: 4G6M)





Figure 8 Docking poses and interaction pattern of selected targets. A: Fibronectin; B: Interaction with fibronectin; C: Interleukin-6 (IL-6); D: Interaction with IL-6; E: Tumor necrosis factor- α (TNF- α); F: Interaction with TNF- α ; G: Interleukin-1 β (IL-1 β); H: Interaction with IL-1 β ; I: Transforming growth factor-beta 1 (TGF- β 1); J: Interaction with TGF- β 1. The docking pose of targets selected based on the binding affinity and RMSD \leq 3.0 Å interacted with Tiliroside. TGF- β 1: Transforming growth factor-beta 1; TNF- α : Tumor necrosis factor- α ; IL-6: Interleukin-6; IL-1 β : Interleukin-1 β .



Figure 9 Effect of Tiliroside on renal fibrotic markers in experimental rats. A: Fibronectin; B: Transforming growth factor-beta 1. All results were statistically assessed by one-way ANOVA and Tukey's *post hoc* test. ${}^{a}P < 0.05$ vs Control; ${}^{b}P < 0.01$ vs STZ. GB: Glibenclamide; TIL: Tiliroside; STZ: Streptozotocin; FN: Fibronectin; TGF- β 1: Transforming growth factor-beta 1.

been prevented.

A significant reduction in insulin levels was observed after STZ treatment, and these findings are consistent with previous research[32]. Interstitial atrophy and vasodilated atrophic alterations in the glomeruli and tubules contribute to an increased kidney weight as diabetes progresses[33]. HOMA-IR is calculated to evaluate resistance to insulin by the animals. A high HOMA-IR value indicates high resistance to insulin. The consumption of food and water was considerably greater in the diabetic group, which may be accounted for by the tissues' reduced ability to use glucose. This impairment causes raised levels of glucose elimination, which is constantly conducive to overeating. In the current study, Til treatment considerably reduced the intake of water and food without altering the BW. In addition, it also reduced the HOMA-IR value and kidney weight. Til treatment increased the level of insulin which is in correlation with the HOMA-IR value. Additionally, an elevation in BUN could indicate a decline in glomerular filtration rate following the onset of diabetes. In reality, both high BUN and high SCr levels are indicators of DN, but BUN is a more accurate indicator for kidney damage[34]. Uric acid is regarded as an accurate indicator for renal function. One of the main characteristics of DN is increased uric acid levels[35]. Increased levels of NAG and U-mAlb are regarded as biomarkers of DN[36]. In this study, the levels of BUN, uric acid, NAG, SCr, and U-mAlb were down-regulated in Til-treated animals in comparison to STZ-treated control animals.

It is important to boost both enzymatic and non-enzymatic antioxidants in the body to counteract oxidative stress, which is the main reason causing the challenges to occur[37]. Antioxidant enzymes, such as GPx, SOD, and catalase (CAT), are the most important components of the defense system and actively contribute to ROS homeostasis. SOD is engaged in the catalysis of superoxide radical degradation, whereas GPx and CAT enzymes catalyze the decomposition

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a279(A)

Cys293(A)

283(A)

Figure 10 LigPlot interactions of selected targets with Tiliroside in 2D visualization. A: Fibronectin; B: Interleukin-6; C: Tumor necrosis factor-or; D:

Arg27

WHY Leu280(A)

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Will

Asn292(A)

دم) کیلللار Cys389(A)

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Interleukin-1β; E: Transforming growth factor-beta 1. TGF-β1: Transforming growth factor-beta 1; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IL-1β: Interleukin-16



Figure 11 Effect of Tiliroside on renal histopathology of experimental rats. Control rats exhibited a healthy tissue appearance with normal histoarchitectures (Group I). The streptozotocin (STZ)-provoked diabetic nephropathy (DN) animals showed shrinkage and necrosis of glomeruli (black arrows), renal tubular epithelial cell damage (blue arrows), edema (green arrows), and neutrophil infiltration (yellow arrows) (Group II). Tiliroside (Til) at three different doses i.e., 25, 50 mg/kg, and positive control Glibenclamide (GB) 600 µg/kg treated Diabetic nephropathy (DN) animals treated with Tiliroside (Til) at different doses, i.e., 25 and 50 mg/kg, and the positive control Glibenclamide (GB) 600 µg/kg demonstrated reduced histological changes such as reduced edema, glomerular and tubular damage, and neutrophil infiltration in renal tissues (Groups III and V, respectively). GB: Glibenclamide; TIL: Tiliroside; STZ: Streptozotocin.

of H₂O₂ to create oxygen and water. GSH reductase catalyzes the conversion of GSH disulfide to GSH sulfhydryl, which is essential for avoiding oxidative stress^[38]. The reduction in antioxidant enzyme activities in diabetic rats has been demonstrated in several studies, and it may be caused by prolonged hyperglycemia-induced ROS production that leads to the decline of antioxidants[39]. Potentially effective treatment options for DN include inhibiting the generation of AGEs, suppressing receptor for AGEs (RAGE) expression, or blocking RAGE downstream signaling[40]. In the present investigation, the levels of antioxidant enzymes including SOD, GSH, and GPx were elevated on treatment with Til. The level of ROS was reduced. This demonstrates the antioxidant properties of Til. In addition, the level of AGE was also reduced in Til-treated animals in comparison to the treated control group. This indicates that Til can be a potent therapeutic for treating DN.

The occurrence of DN is influenced by inflammatory cytokines including IL-6, IL-1β, and TNF-α. Additionally, IL-1β is speculated to contribute to the development of intraglomerular hemodynamic abnormalities linked to prostaglandin production. Increased fibronectin levels, accelerated mesangial cell proliferation, altered extracellular matrix dynamics, and increased endothelial permeability are all effects of IL-6[41]. The onset and progression of renal damage in DN have been linked to TNF- α , a strong pro-inflammatory cytokine. It stimulates swelling, the build-up of the extracellular matrix, and impairment of the glomerular permeability barrier, which results in the onset of albuminuria^[42]. The levels of these inflammatory mediators significantly decreased after Til treatment in diabetic rats, indicating that the drug may have anti-inflammatory properties. During renal damage, fibronectin expression is enhanced. TGF- β , a cytokine that plays a role in the formation of the ECM along with fibronectin, is regarded as the most effective pro-fibrotic molecule. Even though TGF- β and its three isoforms exist, TGF-1 β is known to play a part in renal fibrosis. The expression of TGF-1 β is elevated in DN[43]. Til treatment was successful in downregulating the levels of fibronectin and TGF-1β.

This study has several limitations, such as the absence of full replication of human DN in the *in vitro* model and a specific lack of capture of all aspects of the disease in the *in vivo* model. Studies of Til treatment have been based on specific dosages and durations, there is still confusion about the precise molecular mechanisms, and in silico docking analysis only provides insights into molecular interactions. There is much value in translating animal models to human clinical practice, and larger sample sizes would be able to provide more robust data in the future.

Our histological findings showed injuries to renal tubular epithelial cells, shrinkage and necrosis of glomeruli, swelling, and neutrophil infiltration in STZ-treated rats, which were consistent with the biochemical data and those from other investigations. Reduced glomerular and tubular injury, edema, and neutrophil penetrations in the renal tissues

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were all seen as a result of Til therapy in a dose-dependent manner. Also, the molecular docking analysis confirmed that there is a good interaction between Til and the targeted biomarkers including fibronectin, IL-6, TNF- α , IL-1 β , and TGF- β 1. Specifically, Til highly interacted with TGF-β1.

CONCLUSION

Our research showed that Til reduced inflammatory mediators, blood glucose levels, and oxidative stress, enhanced antioxidants, and inhibited ROS to attenuate the STZ-induced DN in rats. These results validated the suggestion that Til could be a promising treatment for DN. However, further research will be required in the future to produce further evidence of the benefits of Til in DN.

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FOOTNOTES

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