DIOSMIN ATTENUATES CHRONIC KIDNEY DISEASE BY INHIBITING TGF BETA EXPRESSION

Authors

Deepthi Rayilla[1], Suhasin Ganta[2]

[1,2] Department of Pharmacology, GITAM School of Pharmacy, GITAM Deemed to be University, Visakhapatnam 530045, Andhra Pradesh, India.

*Corresponding author:

Suhasin Ganta

Department of Pharmacology, GITAM School of Pharmacy, GITAM Deemed to be University, Visakhapatnam 530045, Andhra Pradesh, India.

Email: deepthirayilla@gmail.com

Abstract

Chronic kidney disease is a non-communicable disease with growing prevalence rates. It usually affects the adult and aging population. Its prevalence is higher in South Asian people because of the higher incidence of metabolic abnormalities. Decreased glomerular filtration rates and damage to the renal structure are the characteristic features of the disease. Controlling the causative factors is the only management strategy to halt its progression to end-stage renal failure. The lack of therapeutic drugs for CKD makes it an essential area of research. Flavonoids are polyphenolic molecules currently investigated primarily because of their multiple pharmacological activities. This study aimed to investigate the renoprotective properties of diosmin in adenine-induced chronic kidney disease. Diosmin is proven to be a renoprotection agent against the experimental model through its anti-inflammatory and anti-fibrotic properties.

Keywords: Chronic kidney disease, TNF α, TGF β1, Inflammation, Fibrosis

Introduction

Chronic kidney disease is a non-communicable disease affecting the worldwide population. Its prevalence is high among Asian people because of the higher incidence of diabetes, hypertension, and obesity among them. According to global estimates, the prevalence of chronic kidney disease

(CKD) is between 11.7 to 15.1%, and the number of patients with end-stage renal failure requiring renal replacement therapy is around 7 million. Although diabetes and hypertension increase the prevalence, other risk factors contributing to CKD include aging, infections, environmental toxins, and herbal toxins[1, 2].

CKD can be defined as chronic kidney damage characterized by albuminuria (proteinuria) or reduced glomerular filtration rate for atleast 3 months. Progression of CKD may result in complications such as anemia, renal osteodystrophy, cardiac remodeling, and vascular calcification[3]. Cardiovascular deaths among CKD patients can be up to 10 times higher than in the general population[4]. Urinary creatinine and albumin (proteins) are considered important diagnostic criteria for assessing the extent of glomerular injury in CKD[5, 6]. According to published data, albumin levels in urine are higher among CKD patients with various etiologies and increase the risk of end-stage renal failure[7].

Oxidative stress, chronic inflammation, and apoptosis contribute to renal fibrosis. Persistent inflammation causes the release of profibrotic cytokines that contribute to tubular atrophy and interstitial fibrosis. Tumor necrosis factor –alpha (TNF α) is an important proinflammatory mediator that increases the expression of transforming growth factor-beta 1 (TGF β1), resulting in extracellular matrix accumulation, fibrosis, and loss of kidney function[8, 9]. Thus developing therapeutic molecules that can prevent the release of proinflammatory and profibrotic cytokines may reduce the progression of renal damage in CKD.

Phytochemicals, particularly flavonoids, are known to control and treat various diseases. They possess free radical scavenging, antioxidant, and anti-inflammatory properties crucial in attenuating renal injury and fibrosis[10]. Excess adenine is metabolized to 2, 8- Dihydroxy adenine filtered through glomerulus but accumulates in renal tubules and generates reactive oxygen species initiating inflammation and fibrosis[11].

Diosmin, a citrus flavonoid glycoside, is currently investigated in this study for its protective actions against adenine-induced chronic kidney disease. Our previous study results have shown that diosmin has alleviated renal injury in adenine-induced CKD by inhibiting oxidative stress and lipid peroxidation[12]. This study aimed to investigate the role of diosmin in helping renal inflammation and fibrosis in CKD rats.

Chemicals

Diosmin and adenine were procured from Sigma Aldrich, India. Losartan Potassium from Med Chem laboratories, Hyderabad, India. TNF α Rat ELISA kit (Abcam lab.), TGF β1 ELISA kit (Sigma Aldrich, India), FITC anti-human LAP (TGF-β1) Antibody (BioLegend), Remaining Biochemistry kits (Anamol Lab Pvt. Ltd.)

Animals

Male Sprague Dawley rats (around eight weeks of age) weighing approximately 200 grams were procured from Jeeva Life Sciences Pvt. Ltd., Hyderabad, India. The animal study is carried out with prior approval of the animal ethical committee for research. The rats were placed in standard polypropylene cages maintaining room temperature around 24 ± 2° C and a 12hrs light and dark cycle. The rats were allowed to feed a regular rat chow diet and had access to tap water. The was conducted according to CPCSEA guidelines with approval (CPCSEA/IAEC/JLS/18/07/22/008)

Experimental design

Rats were randomly divided into five groups (n=6). The drugs were given by oral gavage.
Group-1: Normal Control group was treated with vehicle (0.5% sodium CMC)
Group-2: Adenine Control group was treated with 200 mg/kg adenine suspended in 0.5% sodium CMC
Group-3: Standard Control group was treated with 200mg/kg adenine and 20 mg/kg Losartan Potassium in 0.5% sodium CMC simultaneously
Group-4: Diosmin Low dose group was treated with 200mg/kg adenine and 100mg/kg diosmin suspended in 0.5% sodium CMC simultaneously
Group-5: Diosmin high dose group was treated with 200mg/kg adenine and 200mg/kg diosmin suspended in 0.5% sodium CMC simultaneously
The dosing was administered at the same time of the day and continued for up to 28 days.
At the end of the 28th day, rats were placed in metabolic cages, and 24-hour urine samples were collected to analyze urinary albumin and creatinine. Rats were anesthetized with a ketamine and xylazine combination and sacrificed. Kidneys were dissected; surrounding tissue was cleaned and

stored in the ice-cold buffer for cytokine estimations. A small portion of the kidney was preserved in 10% formalin for histopathology studies.

Urinary albumin estimation

The urinary albumin was estimated with a commercially available kit containing standard albumin. 1000 µl of working reagent was added to 10 µl of standard control albumin, urine sample, or blank solution and incubated for 5 min. The contents were transferred to cuvettes, and optical density was measured at 610 nm.

Urinary creatinine estimation

The urinary creatinine was estimated with a commercially available kit by the Jaffe method. 50 µl of standard albumin or urine sample was added to 1000 µl of working reagent. The contents are mixed well, and the optical density of orange colored product was measured at 500 nm[13].

Histopathology using Masson Trichome staining

2 µm kidney sections were prepared from formalin-fixed tissue and were stained with Masson trichome for observing collagen formations that indicate accumulation of extracellular matrix. The section slides were observed under 100 X of the microscope.

Estimation of renal TNF α and TGF β1

Renal tissue homogenate in buffer was prepared, and the levels of the proinflammatory cytokine, TNF α, and profibrotic cytokine, TGF β1, were estimated by standard ELISA kits.

Expression of TGF β by flow cytometry

A single cell suspension from renal tissue was prepared before flow cytometry. Briefly, renal tissues were sliced, macerated, and filtered. The rest was centrifuged, and the supernatant was discarded and fixed with ethanol. 20 µl of anti-human LAP (TGF-β1) antibody was added to a cell suspension of 10^5 cells/ml. After 30 min incubation, the cells were analyzed by FL-1 channel of

flow cytometry for expression of TGF β1 mRNA. The number of kidney cells expressing the marker was measured [14, 15].

Statistical analysis

The data were expressed as mean ± SEM and analyzed using Graph pad Prism software version-9.5. The data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests.

Results

Table-1: effect of diosmin on urinary albumin and creatinine

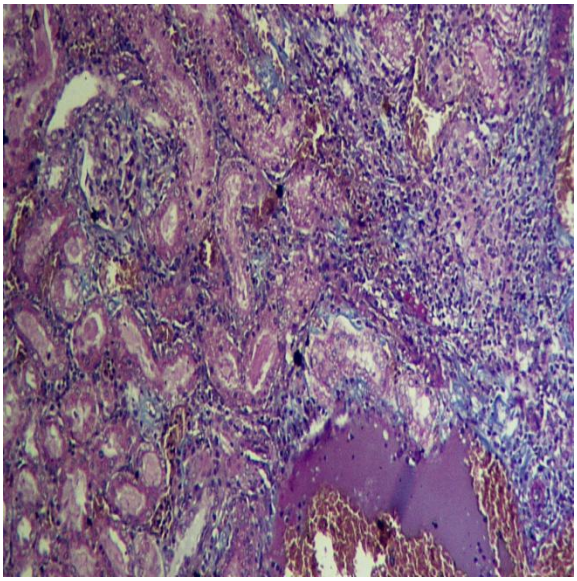
S. No.	Treatment group	Urinary albumin (mg/dl)	Urinary creatinine (mg/dl)
1	Normal control	4.80 ± 0.264	12.79 ± 0.259
2	Adenine control	8.13 ± 0.709	5.13 ± 0.416
3	Ade + Losartan 20 mg/kg	4.66 ± 0.611	10.4 ± 0.529
4	Ade + Diosmin 100 mg/kg	6.40 ± 0.529	7.3 ± 0.360
5	Ade + Diosmin 200 mg/kg	5.50 ± 0.50	9.30 ± 0.20

Data are represented as mean ± SEM. Urinary albumin levels were doubled in adenine 200 mg/kg treated rats compared to normal control group rats. In comparison, urinary excretion of creatinine was halved in adenine-treated rats. The levels were normalized with diosmin treatment.

Histopathology of rat kidney tissue

Masson trichome staining shows the collagen levels in bluish-green color. The collagen content in adenine 200mg/kg treated rats was significantly high, seen as a bluish-green appearance in renal tissue slide. The collagen formation was reduced in diosmin-treated adenine rat groups. The following Fig-1 shows the histopathological results of diosmin.

A **B**



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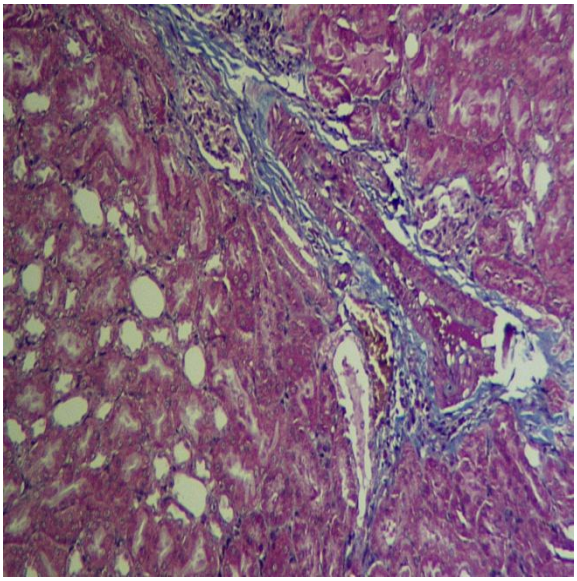

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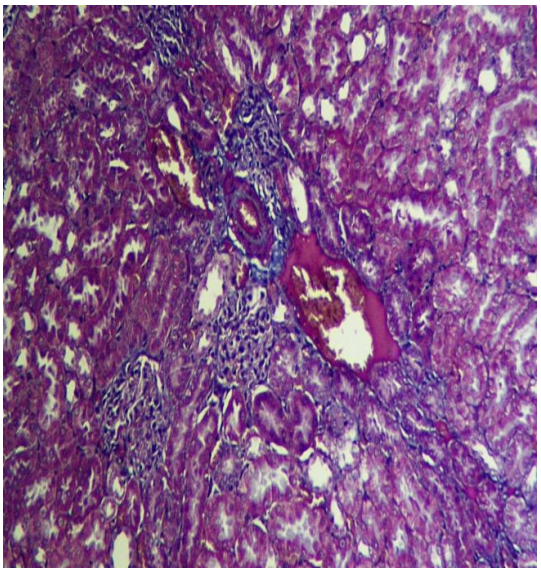

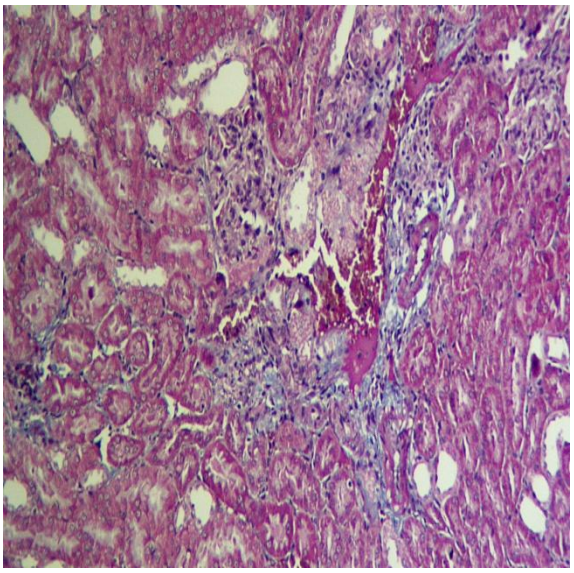

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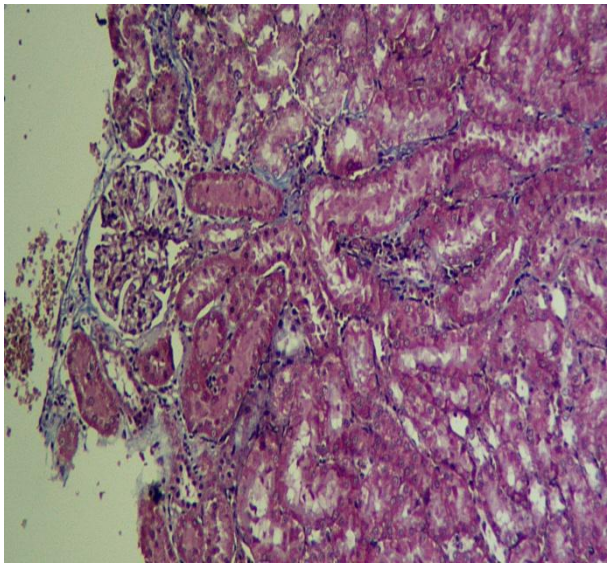


Fig -1: Masson trichome staining of rat kidney sections

A. Normal control B. Adenine control C. Ade + Losartan 20 mg/kg

D. Ade + Diosmin 100 mg/kg E. Ade + Diosmin 200 mg/kg

Table-2: Measurement of TNF α and TGF β1 levels in rat kidney using ELISA

S. No.	Treatment group	TNF α (pg/ml)	TGF β1(pg/ml)
1	Normal control	101 ± 0.070	6.5 ± 2.145
2	Adenine control	622 ± 1.414	49 ± 1.414
3	Ade + Losartan 20 mg/kg	163 ± 2.322	20 ± 0.707
4	Ade + Diosmin 100 mg/kg	325 ± 1.212	36 ± 1.235
5	Ade + Diosmin 200 mg/kg	249 ± 4.321	29 ± 0.101

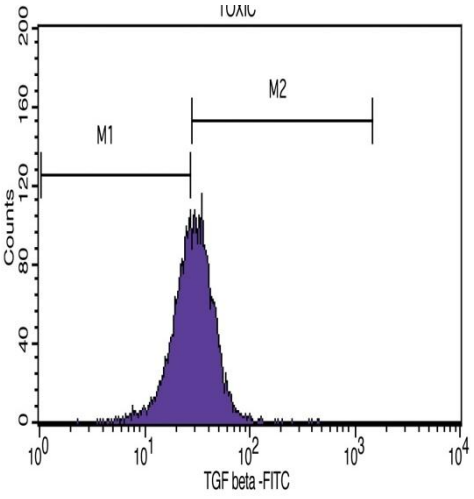
Data are represented as mean ± SEM. Adenine has significantly increased the cytokine levels in rat kidneys. TNFα levels were increased up to 6 folds by adenine compared to the normal vehicle control group. The levels of transforming growth factor β1 have been raised up to seven folds. Diosmin has significantly decreased the levels compared to adenine control rats.

Table-3: effect of diosmin on TGF β1 mRNA expression in rat kidney

S. No.	Treatment group	% number of cells
1	Normal control	3.5 ± 2.1
2	Adenine control	59.2 ± 4.2
3	Ade + Losartan 20 mg/kg	22.6 ± 1.3
4	Ade + Diosmin 200 mg/kg	26.7 ± 3.4

Data are represented as mean ± SEM. The percentage number of cells expressing TGF β1 mRNA is determined by flow cytometry. It has been observed that diosmin has increased the expression of fibrotic mediator up to 55 times compared to normal control group. The levels are halved by diosmin.

A



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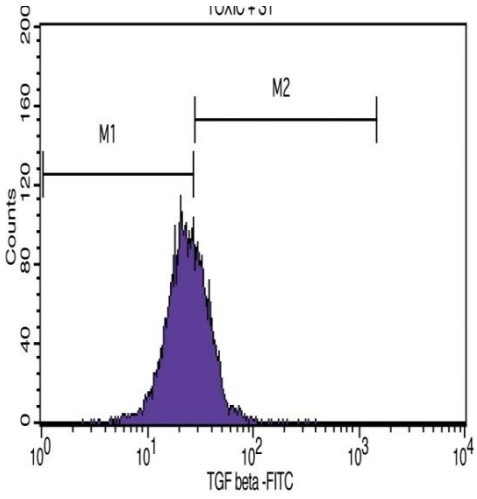

Fig 2: Flow cytometric measurement of TGF β1 expression in rat kidney

A. Adenine 200 mg/kg treated group B. Ade + Diosmin 200 mg/kg treated group

Discussion

Diosmin is flavonoid rhamnoglucoside extracted from citrus fruits. It is commercially available for the treatment of chronic venous insufficiency[16]. It possesses multiple pharmacological effects and is known for its antioxidant, free radical scavenging, anti-inflammatory, antimutagenic, and anticancer properties[17]. Chronic kidney disease has inflammatory pathogenesis, and the phytochemical was selected based on its anti-inflammatory potential. The current study was carried out to investigate diosmin's beneficial effects on CKD.

Adenine is metabolized to uric acid by xanthine oxidase and excreted during normal conditions. Excess intake of adenine causes the deposition of its metabolites in renal tubules and initiates oxidative stress-induced inflammation, apoptosis, and fibrosis. Chronic administration of low doses can produce renal changes similar to human CKD. The model is preferred over the 5th/6th nephrectomy model as it doesn't require surgical modifications that interfere with CKD development. Additionally, adenine-induced CKD rat models produce cardiovascular and other complications generally seen in humans[18, 19, 20]. Thus, the model was selected for the reasons mentioned above.

Albuminuria, a sign of kidney injury, is now used as a biomarker in chronic kidney disease and its associated cardiovascular complications[21]. Urine creatinine is a standard parameter for glomerular filtration rate assessment[22]. Adenine treatment has significantly increased the excretion of albumin and decreased creatinine in urine compared to normal. Administration of diosmin has recovered the ranges as shown in table-1.

Collagen formation in the renal cortex and medulla indicates renal fibrosis that can be observed through Masson trichome staining[23]. Adenine has increased the collagen fibers in adenine-treated kidneys, which might be due to the release of transforming growth factor β1 in response to inflammation produced by adenine. The increase in TNF α and TGF β1 correlate with the amount of bluish-green fiber appearances in stained kidney sections, as shown in Fig-1, table-2. The inference is further confirmed with flow cytometric analysis of rat kidney homogenate against Anti-TGF β1 antibody. The number of cells expressing TGF mRNA was higher in adenine-treated rats (59.2 ± 4.2) than in normal control rats (3.5 ± 2.1). Diosmin 200 mg/kg has lowered the percentage

cell number to (26.7 ± 3.4) as shown in table-3 and fig-2. We are further investigating the protective effect of diosmin on heart and aortic structures as an extension of the current study.

Conclusion

Administration of adenine 200 mg/kg has induced a progressive kidney disease characterized by high albumin and low creatinine ranges in urine. The proinflammatory cytokine levels and fibrotic growth factor, TGF β1 levels, were drastically increased with adenine, proving it a reliable model for CKD. Diosmin has alleviated adenine-induced progressive kidney disease through its anti-inflammatory and anti-fibrotic mechanisms. Hence, diosmin can be proposed as a renoprotective molecule because of above mentioned mechanisms. Further investigations on this flavonoid are warranted.

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Conflict of Interest

None

Funding

Self

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