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# *Boerhavia diffusa* attenuates podocyte injury in rats with adenine induced chronic kidney disease by enhancing nephrin expression

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**Abstract**: Nephrin is a transmembrane protein that maintains the slit diaphragm of renal podocyte. In chronic kidney disease (CKD), podocyte effacement causes damage to glomerular basement membrane barrier leading to proteinuria. *Boerhavia diffusa*, (BD), an Ayurveda herb, is used in treatment of various diseases particularly in relation to the urinary system. This study attempts to evaluate the effect of ethanolic extract of BD on the expression of nephrin in adenine induced CKD rats**.** CKD was induced in Wistar albino rats using adenine (600/mg/kg, orally for 10 days). CKD rats were treated with BD (400/mg/kg/) and pirfenidone (500/mg/kg) orally for 14 days. The kidneys were harvested from euthanized animals and processed for histopathology, electron microscopy and immunohistochemistry, gene and protein expression of nephrin. Diseased rats treated with BD and pirfenidone showed reduction in the thickening of renal basement membranes and reduced haziness in brush border of PCT and glomeruli. Nephrin gene and protein expressions were higher in BD and pirfenidone treated group when compared to the disease control group. The structural and functional damage brought on by adenine-induced nephrotoxicity was countered by protective action of BD by up regulating the expression of nephrin. Therefore, BD can be utilized as a nutraceutical for the prevention and treatment of CKD.

**Keywords**: *Boerhavia diffusa*, chronic kidney disease, nephrin, glomerular basement membrane, adenine.

## INTRODUCTION

Chronic kidney disease (CKD) is a collective term for several diverse kidney illnesses identified through abnormal urine results, blood biochemical analyses, diagnostic imaging, or pathological findings. CKD is defined as a decline in kidney function characterized by proteinuria for three months or longer or decreased glomerular filtration rate. The prevalence of CKD in the US was estimated to be 14.8% between 2011 and 2014, and in 2014, there were 3% more people on the kidney transplant waiting list than in 2013 (Nagata *et al*., 2010, Gansevoort *et al*., 2013, Saran *et al*., 2016). CKD patients are more prone for congestive heart failure, coronary artery disease, and sudden death. (O'neill *et al*., 2013).

The components of glomerular filtration barrier are endothelium and basement membrane of glomerulus and foot processes of podocytes, (Ronco *et al*., 2007). A membrane known as the slit diaphragm (SD) connects neighboring podocytes' foot processes so that they can interdigitate (Sever *et al*., 2007). The glomerular basement membrane (GBM) and SD act as barriers to stop the filtration of plasma macromolecules like proteins. Podocytes go through a process called effacement after being wounded, during which they lose their structure, become diffuse, spread out, and lessen the effectiveness of the filtration barrier (Wiggins *et al*., 2007, Philippe *et al*., 2008). The actin filaments present in the foot processes of podocytes dynamically reorganize themselves in response to the filtration requirements. During effacement this actin cytoskeleton is broken down. However, not all proteinuria instances exhibit podocyte effacement and it is yet unclear how structure and function interact (Kestila *et al*., 1998, Garg *et al*., 2007). Moreover, depletion of podocytes is associated with the advancement of CKD and glomerular sclerosis.

Nephrin is a transmembrane adhesion protein in the SD that is expressed by the NPHS1 gene. Nephrin interacts with the cytoplasmic tail of the SD and plays a role in the interaction between the SD and the cytoskeleton of the foot processes. Nephrin plays a structural purpose in addition to playing a part in podocyte signaling. It is a member of the immunoglobulin super family. It is a single-spanning protein that engages in intracellular and extracellular interactions with Trans and cis nephrin proteins. Finnish type of congenital nephritic syndrome, originally discovered in 1998, has been linked to nephron abnormalities brought on by NPHS1 gene mutations (Santin *et al*., 2011). The podocyte foot processes and slit diaphragms fail to develop as a result of a single gene mutation and there will be severe proteinuria in utero.

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A number of CKD models have been reported (Becker *et al*., 2013, Ortiz *et al*., 2015, Glastras *et al*., 2016). Adenine model isa commonly used CKD model. Adenine is a purine base. Adenine on oral administration metabolizes to form poorly soluble 2,8 dihydroxy adenine (DHA) which is excreted in the urine. Excess intake of adenine produces excessive production of DHA and stored in tubular epithelial cells leading to inflammation and subsequent tubulointerstitial fibrosis. An adenine diet is utilized to exacerbate the condition in CKD model (Diwan *et al*., 2018).

CKD is regarded as a serious health issue due to its frequency and bad prognosis. New therapeutics is thus required to treat this illness. *Boerhaavia diffusa* (BD) L., a member of the Nyctaginaceae family, is an indigenous ayurvedic remedy for urinary disorders (Apu *et al*., 2012, Malla *et al*., 2021). In addition to being a medicinal plant, BD is used as a green leafy vegetable in numerous Asian countries due to its nutritional properties. BD is a good source of nutritional supplements as it contains 15 amino acids (6 essential) in the whole plant and 14 amino acids (7 essential) in the roots along with palmitate acetate, behenic acid, arachidic acid (6.3%), saturated fatty acids (38%), vitamins C, B3, and B2 and calcium. BD contains various categories of secondary metabolites like flavonoid glycosides, isoflavonoids (Kaur *et al*., 2019, Gaur *et al*., 2022). BD is employed as an adaptogenic agent and used in treatment of various ailments, such as dyspepsia, jaundice, spleen enlargement, and stomach pain. Pharmacological studies on BD have revealed that it has cardiotonic, anti-diabetic, antihypertensive, anti-immunosuppressive, anticonvulsant, hepatoprotective, antibacterial, antiproliferative, anti-covid 19 activity and anti-estrogenic effects. BD's antimitotic activity is established in mouse and rat monocytic, lymphoblastoid, fibroblastic, and erythroleukemic cell lines (Munasinghe *et al*., 2001, Pari *et al*., 2004, Manu *et al*., 2009, Agarwal *et al*., 2010, Kaur *et al*., 2011, Srivastava *et al*., 2011, Sinan *et al*., 2021, Kaviya *et al*., 2022, Rutwick *et al*., 2021). This study attempts to evaluate the role of BD in the modulation of nephrin expression in the renal tissues of CKD induced rats, which plays an important role in the maintenance of glomerular integrity.

## MATERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Ethics Committee of the Chettinad Hospital and Research Institute (IAEC 1/proposal: 74/A.Lr: 54/Dt: 06.05.2022), and all procedures were carried out according to the CPCSEA's Guidelines for Care and Use of Animals in Scientific Research.

### *Animals*

200-250g Wistar male albino rats were employed. After acclimatization, rats were divided into five groups as described below (table 1):

The animals were housed in normal lab conditions, with 12-hour light/dark cycle, a constant temperature of 25±2 ˚C and a relative humidity of 60. 5%. The animals were provided water and food ad libitum. At the end of the study animals were euthanized with overdose of anesthesia (Halothane by inhalation). Kidneys were dissected from euthanized animals.

### *Induction of CKD*

Group 3, 4 and 5 animals were administered adenine (600mg/kg/day) orally for 10 days using 0.5% carboxyl methylcellulose (CMC) as a vehicle. At the end of 10 days blood urea (>40mg/dl) and creatinine (>1.2mg/dl), levels were used to confirm the CKD.

### *Ethanolic extract of BD*

The plant BD was purchased on August 2021 from the local market and authenticated by Dr. S. Karpagam, Department of Botany, Associate professor and Head, Queen Mary's college, Chennai-600004. A voucher specimen (BOTANYQMC/2021/1101) was deposited in the Department of Botany, Queen Mary's College.

The ethanolic extract of the plant material was prepared as per the method described by A. Prathapan *et al*., 2017 with a few minor modifications. In a nutshell, fresh whole plants were air-dried and then ethanol was added and agitated for 6 hours at room temperature (27±1˚C) to extract the compounds. To make the solvent colorless, the extraction procedure was done 3 times. A rotavapor was used to condense the supernatant under reduced pressure before filtering it through Whatman No. 1 filter paper. It was lyophilized and maintained at 4°C until use. The yield of the extract was found to be 4.33% w/w.

Group 2 and 4 animals were administered ethanolic extract of BD (400mg/kg/day) orally for 14 days using 0.5% CMC as a vehicle.

Nephrin polyclonal antibody (raised in rabbit) and adenine was purchased from Abcam (Cambridge, UK) and Sisco Research Laboratories Private limited (Mumbai, India) respectively.

### *Histopathology*

Formalin fixed kidney tissues were processed, 5microns thickness section were taken and stained with periodic acid Schiff (PAS). The photomicrographs taken using Carl Zeiss microscope and Axiocam camera.

### *Immunohistochemistry*

The sections were immersed in hydrogen peroxide for 10 minutes to stop any naturally occurring peroxidase activity in the tissues. Following a 1hour bovine serum albumin blockade, sections were incubated with primary polyclonal antibody for 1 hour at room temperature against nephrin. The sections were then incubated with an HRP-conjugated secondary antibody for two hours and coloured using 3, 3'-diaminobenzidine tetrahydrochloride.

***Western blot analysis***

The protein contents were analyzed in rat kidney samples that were homogenized in RIPA lysis buffer. The entire proteins were incubated in boiling water for 10 minutes. The proteins were separated using 12% SDS-PAGE and electrophoretically transferred in equal amounts to polyvinylidene difluoride membrane. Methanol was included in the transfer fluid to preactivate the membranes. After being blocked with 5% skimmed milk for two hours, the membranes were treated overnight at 4°C with the primary polyclonal antibody against nephrin. After cleaning the membranes, a secondary antibody directed against rabbit IgG was added, and it was incubated for an additional 60 minutes at room temperature. The immunoreactive proteins were discovered with the use of an enhanced chemiluminescence tool. The immunoreactive bands were detected using the Phototope HRP Western Blot Detection kit.

***RT-PCR***

Using RT-PCR, gene expression was assessed. To assess the mRNA expression of the genes encoding nephrin, total RNA was extracted from individual rat kidneys. cDNA was made in 20microliterPCR experiments using HiScript II QRT SuperMix and kept at 20°C until analysis. The SYBR-Green reaction kit was used to conduct RT-PCR amplification using the ABI Step One Plus apparatus. table 2 contains the list of the RT-PCR primers. Comparative cycle threshold (CT) analysis was utilized to analyze the data that was acquired. The 2-ΔΔCt method was used to determine the gene's expression.

***Electron microscopic study***

The kidney tissues were fixed in glutaraldehyde (2.5%) in 0.1 M cacodylate buffer, pH 7.2-7.4 for 24 hours. The tissues are, postfixed in 1% osmium tetroxide, dehydrated through a graded series of ethanol, cleared in propylene oxide, and then impregnated with Araldite embedding medium. Uranyl acetate and lead citrate were used to stain the ultrathin slices. While the FEI, TECNAI T20G2 TEM attached with EDX was used to photograph the extremely thin slices of kidney.

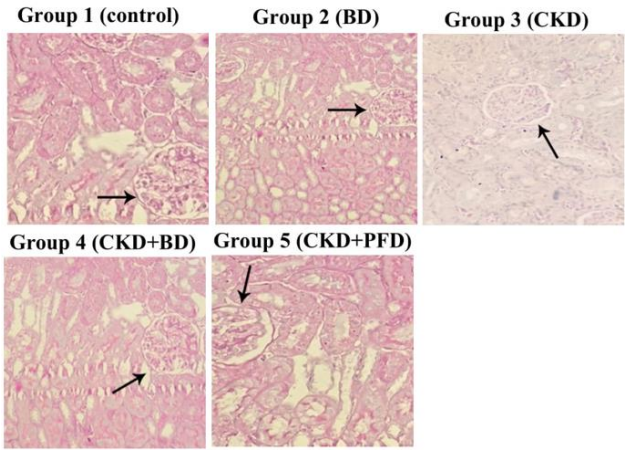
## STATISTICAL ANALYSIS

A quantitative data were expressed as mean ± standard deviation. One way analysis of variance (ANOVA) was used to compare the difference between the groups. Data analysis was performed using SPSS (version 26.0.). P value <0.05 was considered as significant.

## RESULTS

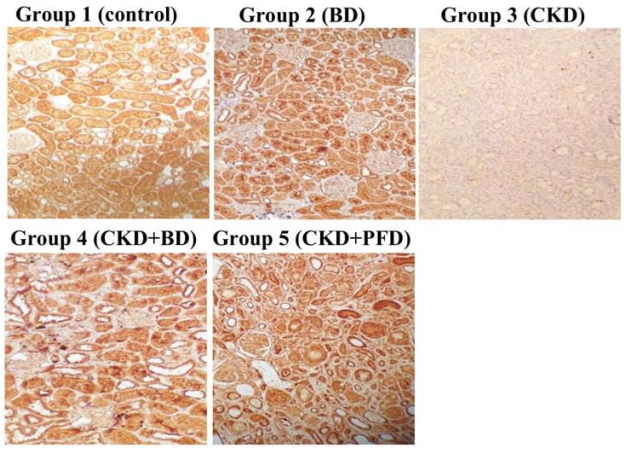
***Kidney histopathology - PAS (Periodic acid Schiff stain)***

Groups 1 and 2 showed normal basement membrane. However, group 3 animals displayed thickening of the renal basement membrane. In group 4 the BD treatment showed near normal renal basement membrane (fig. 1).




Magnification 400X.In group 1, arrow denotes normal basement membrane. In group 3 arrow mark denotes the thickening of basement membrane. In group 4 arrow mark denotes the near normal renal basement membrane.

**Fig. 1**: PAS stain




Magnification 100X

**Fig. 2**: Nephrin immunohistochemistry

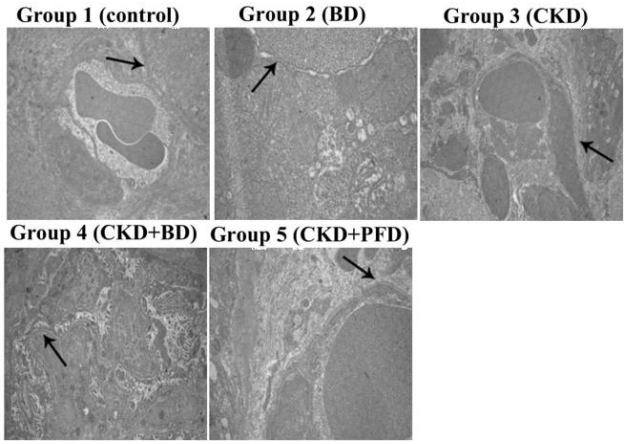
***Immunohistochemistry***

Control animals showed normal expression of nephrin in renal tissue. Group 2 animals treated with BD showed higher expression of nephrin. Group 3 untreated CKD rats displayed less immunopositivity for nephrin antibody than control animals. CKD animals treated with BD and PFD had higher levels of nephrin expression indicating the role of test drugs in the modulation of nephrin expression. (fig. 2)

***Ultra structural changes***

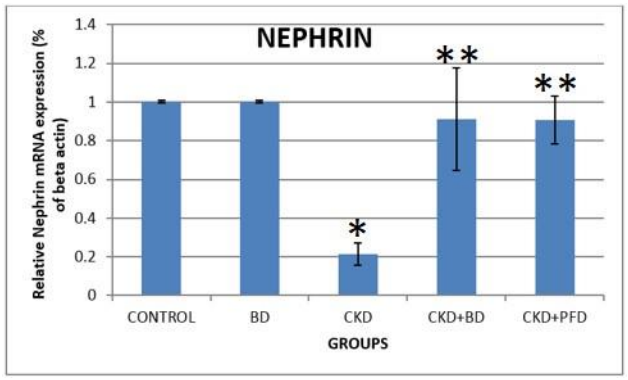
The epithelial cells of PCT and glomeruli are considered normal when the brush border is intact and organelles of cell are normal in appearance. Electron microscopic examination showed detectable changes in the PCT and glomeruli of CKD induced animals. Brush border was

hazy and disturbed, mitochondria were swollen in the PCT and glomeruli and GBM was thick and split. Whereas, the BD treated group animals showed normal mitochondria and nucleus in the PCT and glomeruli (fig. 3).




In group 3, arrow mark denotes the thickening of basement membrane. In group 1, 4 and 5, arrow marks denote the normal renal basement membrane.

**Fig. 3**: Electron micrographs of renal tissues of different groups.




*Significantly different when compared to control,
** Significantly different when compared to group 3, p<0.05

**Fig. 4**: Nephrin gene expression

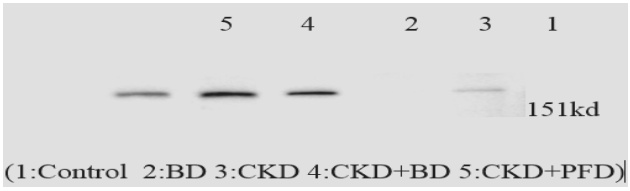



**Fig. 5**: Nephrin protein expression

***RT-PCR and Western blot***

Real-time PCR (fig. 4) and Western blot (fig. 5) investigations revealed that group 3 rats had significantly (P value <0.05) lower nephrin gene and protein expressions when compared to control. Group 4 rats treated with BD showed four-fold increases in the expression of nephrin gene (0.9126±0.265) and protein in comparison with group 3 (0.2159±0.0573) animals. Group 5 showed changes (0.9058±0.1239) similar to group 4 animals (figs. 4-5).

## DISCUSSION

The treatment of kidney illnesses, including chronic renal disease with herbal therapy has a long history. The treatment group displayed an increase in nephrin expression, indicating that BD has a nephroprotective action.The glomerular filtration barrier is made up of endothelial cells, glomerular capillary basement membrane, and podocytes. Podocytes play a crucial role in preserving the structural and functional integrity of this barrier and stop the entry of plasma proteins into the urine by serving as a component of the glomerular basement membrane (Patrakka *et al*., 2009). Numerous studies have shown that proteinuria and podocyte damage and loss are closely related. Delaying the progression of CKD can be accomplished in part by reducing proteinuria and managing blood pressure. In this study, we looked the impact of ethanolic extract of BD on adenine-induced chronic kidney disease rats. We observed that 400mg/kg of BD extract treatment improved renal histopathology and increased nephrin gene and protein expressions. The basement membrane thickening or disorientation of the basement membrane and tubular damage were the most severe effects of adenine-induced CKD rats. In our previous publication monocyte/macrophage infiltration causing damage to the basement membrane of renal tubules were shown to be reduced by the treatment of BD (Sadayan *et al*., 2023).

In podocytes, actin remodelling regulates the podocyte structure which facilitates its adaptation to the filtration needs. A robust actin cytoskeleton, which supports the podocyte's flexibility in the face of changing pressure conditions, is present. The foot processes' cytoskeleton is made up primarily of F-actin-based microfilaments, and F-actin polymerization and depolymerization are crucial processes in preserving the dynamic equilibrium of the podocyte skeleton. ACTN4 protein, a crucial molecule, anchors SD complex to the actin cytoskeleton by cross-linking. Nephrin is a crucial signalling molecule that controls podocyte function in the slit diaphragm. Connection of podocin, a transmembrane protein specific to podocyte to actin cytoskeleton controls the functions of podocytes (Grahammer *et al*., 2013).

Existing litrature displays significant nephroprotective action of BD. Aqueous extract of BD root (200 mg/kg/day for 14 days) exibited nephroprotective action in kidney damage caused by acetaminophen (Pareta *et al*., 2011). In our previous study we have shown that BD at a dose of 400 mgfor 14 days reduce the tubulointesitial fibrosis and glomerulosclerosis in adenine induced CKD by downregulating TGF-β gene and protein expresions (Sadayan *et al*., 2023). In both these studies BD

**Table 1**: Grouping of animals

| Group | Name | Description | Number of animals |
|---|---|---|---|
| 1 | Control | Received only vehicle | 6 |
| 2 | BD only | BD extract only for 14 days | 6 |
| 3 | CKD | 600 mg/kg/day of adenine for 10 days | 8 |
| 4 | CKD+BD | 400 mg/kg/day of BD extract 14 days | 8 |
| 5 | CKD+ pirfenidone (PFD) | PFD solution 500 mg/kg/day 14days | 8 |

**Table 2**: Primer sequence

| S. No. | Gene | Forward primer (5′→3′) | Reverse primer (5′→3′) | Length |
|---|---|---|---|---|
| 1 | Nephrin | ACTCAGGCTGACATCTGGGAT | AGAGCTGGAATGACAGTGATGG | 21<br>22 |
| 2 | β-Actin | GCAGATGTGGATCAGCAAGC | GGTGTAAAACGCAGCTCAGTAA | 20<br>22 |

attentuates histopathological changes caused by acetaminophen and adenine.

Chinese herbal remedy called Huangqi Guizhi Wuwu Decoction contains the herbs *Radixastragal* and *Ramulus cinnamomi*. In the kidney of IgA induced nephropathy, nephrin was dephosphorylated and the podocyte cytoskeleton was compromised. Huangqi Guizhi Wuwu Decoction controlled the AT1R/Nephrin/c-Abl pathway to reduce podocyte cytoskeletal protein damage (Liu *et al*., 2021). HQH (Huaiqihuang Granules) improved nephrin expression in adriamycin-induced nephropathy rats and there by reduced glomerulosclerosis and renal tubulointerstitial lesions. This action was acehived by inhibition of necrosis factor κB signaling pathway (Liu H *et al*., 2017).

Comparable results on the expession of nephrin was displayed by three Chinese medicines: Shen-qi-di-huang (decoction) in adriamycin induced nephropathic rats; Shenyan Xiaobai (granules) in adriamycin induced nephropathic rats; and Gushen Jiedu (capsule) in diabetic nephropathic rats (Chen *et al*., 2011, Cao *et al*., 2019, Zhang *et al*., 2020). Gushen Jiedu capsule was shown to positively modulate the expression of nephrin through Akt pathway and by supressing mitochondrial apoptosis (Zhang *et al*., 2020). The possibility of BD acting on the pathways namely: AT1R/Nephrin/c-Abl pathway, Akt pathway, and necrosis factor κB signaling pathway and supression of mitochondrial apoptosis in modulating the nephrin expression needs further research.

## CONCLUSION

The current study shows ethanolic extract of BD maintains the normalcy of renal basement membrane and increases the expression of nephrin, a protein involved in the preservation of GBM integrity. Therefore, BD having nephroprotective effect can be utilized as a nutraceutical or therapeutic food for the prevention and treatment of CKD. To determine its clinical relevance and therapeutic potential, additional in-depth research is needed.

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