Vitex Negundo extract ameliorates diabetic nephropathy by downregulating Angiotensin II in kidneys of Wistar Rats

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Abstract

Aim To study the effect of *Vitex negundo* in diabetic nephropathy using Wistar rats.

Background Diabetes mellitus is a metabolic disorder highly prevalent in India. With its associated nephropathic complications, it is one of the major disorders to reckon with for the medical fraternity. Diabetic nephropathy is a matter of great concern as, till date, we do not have any treatment to reverse or stop the progression of the disease.

Objective This study is directed towards the remedy of diabetic nephropathy, focusing on the improvement of the diseased condition using a methanolic extract of *Vitex negundo*, which is a popular plant in folklore medicine against kidney ailments associated with diabetes mellitus. Also we tried to find out the probable mechanism of action for such activities and also tried to find out the chemical constituent with their corresponding abundance by GC-MS.

Method Diabetic neuropathy was induced in the wistar rats using streptozotocin and nicotinamide. The aforesaid extract was fed to the rats orally, and the biochemical and urine parameters were assessed. Along with this, the conditions of the oxidative stress of the extract were also assessed. The histological studies and also the estimation of the peptide angiotensin II were carried out via the ELISA techniques.

Result *Vitex negundo* extract improved the biochemical and urine parameters and also resisted the morphological derangements in the histological structure of the nephron and its associated structures by reducing the levels of angiotensin II in the kidney. The probable compounds responsible for such activity can be the compounds with corresponding phenolic groups because such compounds are known to possess maximum antioxidant effects.

Conclusion The study reveals *Vitex negundo* extract's preventive effect on chemically induced diabetic nephropathy by downregulating serum angiotensin II levels. However, various cellular pathways relating to the downstream modulation of angiotensin II pathway are yet to be evaluated.

Keywords Nephropathy, Fibrosis, Inflammation, Angiotensin II, Diabetes Mellitus, *Vitex Negundo*

1. Introduction

According to the International Diabetes Federation (IDF), the global cases of diabetes mellitus were 366 million in 2011, and this number is expected to reach 552 million by 2030 (Glovaci et al., 2019). Diabetes mellitus persistent hyperglycemia causes end-organ malfunction and failure, including the retina, kidney, neurological system, heart, and blood vessels (Alam et al., 2014). Type 1 diabetes is caused by a complete lack of insulin, and it has an autoimmune origin (Kaul et al., 2012). The most common type of diabetes, type 2, affects 90–95% of cases. Insulin resistance is the main cause of type 2 diabetes (Blair, 2016).

Diabetic nephropathy is a leading cause of renal end-stage disease. It thickens the glomerular basement membrane and expands the mesangial matrix, impairing glomerular filtration function (Gross et al., 2004). These haemodynamic factors activate intracellular second messengers such as protein kinase C (PKC), MAP kinase, nuclear transcription factors such as NF-kB and various growth factors such as TGF-β (Soldatos & Cooper, 2008). Glucose-dependent pathways are also triggered, leading to increased oxidative stress, renal polyol production, and AGE build-up (Parchwani & Upadhyah, 2012). These processes eventually lead to increased renal albumin permeability and extracellular matrix build-up, which cause proteinuria, glomerulosclerosis, and, eventually, tubulointerstitial fibrosis (Shumway, 2002). Reactive oxygen species (ROS) and oxidative stress rises due to hyperglycaemia in diabetes. Deoxyribonucleic acid (DNA) damage, renal vasoconstriction, protein oxidation, and lipid peroxidation in cell membrane are all brought on by these ROS. Moreover, the enhanced generation of ROS activate additional metabolic pathways, mainly PKC pathways, AGE formation, TGF-β, and ANG-II. Apart from its function in the onset of diabetic nephropathy, the advanced glycation pathway play a crucial role in developing specific cardiovascular issues such as atherosclerosis, elevated arterial stiffness, and myocardial stiffness.

Commonly known as nirgundi, the medicinal plant *Vitex negundo* (VN) is known to have antioxidative and anti-inflammatory potential (Husain et al., 2022). Belonging to the family verbanaceae, this plant has a tremendous potential against diabetic nephropathy according to folklore medicine (Qu et al., 2023). Methanolic extract of the leaves of VN were prepared and were already studied to have nephroprotective activity (Sahoo et al., 2014). It was studied that the methanolic extract of the plant VN has potential nephroprotective properties against gentamicin induced nephrotoxicity. Therefore the same extract was used in our study against diabetic nephropathy with the same dose. Moreover in this study we will try to establish any link between angiotensin II levels and the activity of the extract against diabetic nephropathy.

2. Materials

2.1 Experimental animals

The male wistar rats, (14-16 weeks, weighing 180-220 g), were used for the experimental study and were housed under a standard environment (22 ± 3 ºC, with a 55 ± 6% humidity and 12:12 h light/dark cycle). Wistar rats were procured from the animal house of Sanaka

Educational Trust's Group of Institutions, Malandighi, Durgapur, West Bengal (Reg. no. 1458/PO/E/11/CPCSEA). Standard pellet diet and tap water under sanitary condition were fed to the animals. All the experimental animal protocols used in this research work were as per the regulations specified by the Institutional Animal Ethics Committee (IAEC), Sanaka Educational Trust's Group of Institutions, Malandighi, Durgapur, West Bengal. The guideline of the Committee for Control and Supervision of Experiments on Animals (CCSEA) Government of India were strictly followed throughout the experimental protocol.

2.2 Chemicals

STZ (streptozotocin, extra pure, 98%, C8H15N3O7, M.W 265.22) and Nicotinamide were purchased from SRL.

Buffer solution: Citric acid and trisodium citrate

AUTOSPAN, Liquid gold creatinine purchased from Arkray.

AUTOSPAN, Liquid gold albumin purchased from Arkray.

AUTOSPAN, urea purchased from Arkray.

Tris HCL buffer

Trichloroacetic acid (TCA)

Tertiary butyl alcohol (TBA)

Sodium carbonate

Ethylenediamine tetra acetic acid (EDTA)

Nitro blue tetrazolium (NBT)

3. Methods

3.1 Preparation of the extract

50 grams of clean dry leaves were taken in a 1000 ml beaker & 800 ml of methanol was added to it. The mixture was transferred to a conical flask and the mouth was sealed. The conical flask was shaken vigorously for 48 hrs at interval of 1 hr. After 48 hrs the green fluid was filtered out and then dried to get the extract. The extract was prepared as a suspension using tragacanth and was administered orally to the animals.

3.2 Procedure for profiling of GC-MS

GC-MS (Gas Chromatography-Mass Spectrometry) analysis was conducted using Shimadzu Triple-Quadrupole GC-MS-MS-TQ8050NX. Samples were analysed on a fused-silica capillary column HP-5MS, 5% Phenyl Methyl Siloxane (length 30 m × 0.25 mm ID. film thickness 250 µm). The analyses were performed by injecting 1.0 µL of the sample at a split ratio of 10:0. The oven temperature was programmed starting at 40 °C, 2 °C/min up to 250 °C. For the identification of the compounds, commercial databases (NIST17R.lib and Wiley) and comparison of spectra with laboratory mass spectra libraries built up from pure substances and MS literature data were used. Identification of the components was also based on their GC retention indices on a polar column, using the homologous series of n-alkanes (C_5–C_{26}) as a reference, and their comparison with those of literature data.

3.3 Study design

At first, all the 30 rats were divided into three groups. On the first day, 2 groups of overnight fasted rats were administered with a single intraperitoneal injection of STZ (55 mg/kg) and Nicotinamide (110 mg/kg). One group of overnight-fasted rats was fed phosphate-buffered saline to maintain normal conditions which served as the control. Measuring fasting blood glucose levels 72 hours after injection allowed for confirmation of diabetes. On the 3rd day, blood glucose level and body weight were measured, and the glucose levels more than 250 mg/dl were diagnosed as having diabetes. On day 65, following the STZ/NA injection, an oral glucose tolerance test was conducted. According to the established process, blood samples were collected from the overnight fasted rats on the 65th day. At the end of the study (day 65), rats were placed in metabolic cages, and urine samples were collected for 24 hours and checked for urine albumin level, blood creatinine and blood urea nitrogen levels were analysed from blood samples. There are two groups of diabetic rats. One group of diabetic rats was given VN extract for 30 days at a dose of 200mg/kg. So, three groups have been identified: normal, diabetic, and treated groups. Finally, at the end of the study, all rats were sacrificed; their kidneys was isolated for histopathological studies, and they were also checked for different oxidative markers (malonaldehyde, superoxidase dismutase) and also angiotensin II level in the serum by Elisa plate reader.

3.4 Determination of metabolic parameters

3.4.1 Blood glucose

Blood glucose levels in each animal group were measured with a glucometer (Dr. Morepen Gluco One, New Delhi). Blood glucose level was measured between 10:30 a.m. and 12:30 a.m.

3.4.2 Body weight

The body weight change in each animal group was observed after the study was completed to ensure that the therapy affected body weight. The body weight of each animal was measured on the first and last days of the experiments.

3.4.3 Oral glucose tolerance test

The oral glucose tolerance test is a commonly used clinical test for diagnosing glucose intolerance and type II diabetes. Food was discontinued the night before the test. The rats were administered glucose (2 g/kg p.o., dissolved in saline). A glucometer was used to assess blood glucose levels in blood samples drawn from the tip of the tail at 0, 30, 60, and 120 minutes after the bolus (Qu et al., 2023, Jude and Gopi, 2021).

3.4.4 Urine albumin

To test urine albumin, animals should be placed in a metabolic cage for 24 hours and their urine was collected. Urine samples were obtained for 24 hrs. The albumin concentration was then determined using the Bromocresol Green, End point assay. For this, we used the AUTOSPAN Liquid Gold albumin kit. We combined the diluted urine with albumin reagent and albumin standard reagent, then evaluated the absorbance in colorimeter (Jude and Gopi, 2021, Saravanan et al., 2023).

3.4.5 Serum creatinine

To determine serum creatinine, we collected blood samples from the tail vein of rats, receiving regular diet ad libitum. Creatinine concentration was then measured using the modified Jaffe's reaction, Initial rate assay. For this, we used AUTOSPAN, a Liquid Gold Creatinine kit. According to this kit, we mixed the blood sample with picrate reagent and sodium hydroxide reagent and tested the absorbance at 2-minute intervals (Abreu et al., 2014).

3.4.6 Blood urea nitrogen

Blood samples were taken from the tail veins of rats fed a typical diet to measure blood urea nitrogen levels. The Urease Berthelot Endpoint assay was used to quantify urea levels. We used a urea kit to accomplish this (*Book: Clinical Chemistry - Theory, Analysis, Correlation (Kaplan and Pesce)*, 2020).

3.4.7 Determination of malonaldehyde (MDA)

After sacrificing the animals, both kidneys should be removed properly. First, the tissue homogenate was processed. In this process, tissue was rinsed with 0.9% ice-cold normal saline then prepared 10% homogenate with PBS. After that, centrifuged tissue homogenates

at 10,000 rpm for 20 min at 4 degrees centigrade and the supernatant was used for the analysis of MDA according to the established process (Besseling et al., 2021).

3.4.8 Determination of superoxidase dismutase (SOD)

By assessing the SOD enzyme's suppression of nitro blue tetrazolium (NBT) photoreduction, SOD activity was ascertained (Hanifa et al., 2019). With a final volume of 3.0 mL, the reaction mixture included 100 µL of crude extract, 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 50 µM NBT, 10 µM riboflavin, and 50 µM NBT. Crude extract was not used in the control reaction. The reaction mixture was subjected to white light for 15 minutes at room temperature in order to initiate the SOD reaction. Using a spectrophotometer, absorbance at 560 nm was measured following a 15-minute incubation period. A single unit (U) of SOD activity was determined to be the quantity of enzyme responsible for 50% of the photochemical reduction of NBT.

3.5 Histopathological assessment

Rats were killed after being anesthetized with ketamine at the end of the experiment. All kidneys were extracted and decapsulated. Right kidney tissue samples were preserved with 10% neutral formaldehyde. Following tissue fixation, they proceeded to tissue processing using various ethanol concentrations. Tissues were prepared and embedded in paraffin before being stained with hematoxylin and eosin. A microtome was used to cut paraffin sections to a thickness of 3-4 micrometres. Hematoxylin and eosin-stained slices were examined. After completing all operations, photographs were analysed under a microscope using Image J software (Wills, 1965).

3.6 Estimation of Serum Angiotensin II using Elisa

Tissue homogenization method was used and the tissue extract samples were used in Elisa plate readers for the estimation of the tissue markers. ELISA plates pre-coated with antibodies specific for rat Angiotensin II were used for the analysis of the expression of the protein. Biotinylated antibodies specific for rat angiotensin II and avidin horseradish peroxidase conjugate were added successively to the wells of each microplate and incubated at 37 c. After washing, the substrate solution was added to each well. The OD was measured spectrophotometrically at a wavelength of 450 nm. The OD value was proportional to the concentration of angiotensin II (Kono, 1978).

3.7 Determination of kw/bw ratio change

After completion of hemodynamic parameters, individual kidney weights were taken in each group divided by the respective animal's body weight and multiplied by 100 to ensure the % change in KW/BW ratio. Formula: Kidney weight (gm.)/ Body weight (gm.) × 100 (Wills, 1965).

3.8 Statistical Analysis

Statistical analyses were performed using Graph Pad Prism 9.0 software (Graph Pad Software, Inc., USA). The values were expressed as means ± standard error of the mean (SEMs) (n = 6). The differences between multiple groups were statistically analysed by using one-way and two-way analyses of variance (ANOVA), followed by post-hoc tests. Values of $P< 0.05$ were considered as statistically significant.

4. Results

4.1 Results on GC-MS profiling

The GC-MS profiling of *Vitex negundo* provides insights into its chemical composition, which is crucial for understanding its pharmacological potential. The GC-MS analysis of *Vitex negundo* revealed a diverse array of compounds, as indicated by the chromatogram and the peak report. A total of 37 compounds were identified, with varying retention times and peak areas, suggesting a complex mixture of phytochemicals. Notable compounds included: Succinic acid - Known for its role in metabolic processes and potential therapeutic effects. 1,6-Dideoxy-l-mannitol - A sugar alcohol that may have implications in diabetes management. 2-Cyclopenten-1-one - A compound with potential anti-inflammatory properties. Butanoic acid, 1-methylpropyl ester - Associated with flavour and aroma, indicating potential applications in food and fragrance industries. Phenolic Compounds - Such as phenol and its derivatives, which are known for their antioxidant properties. The relative abundance of these compounds varied, with some compounds like Hexitol and DL-Lactamide showing significant peak areas, indicating their prominence in the extract. The presence of various phytochemicals in *Vitex negundo* highlights its potential as a source of bioactive compounds. The identified compounds can be categorized into several classes, including: Fatty Acids and Esters: These compounds are often linked to anti-inflammatory and antioxidant activities, which may contribute to the therapeutic effects of *Vitex negundo* in traditional medicine. Phenolic Compounds: The high levels of phenolic compounds suggest that Vitex negundo may possess significant antioxidant properties, which are beneficial in preventing oxidative stress-related diseases. The GC-MS profiling of *Vitex negundo* has successfully identified a range of bioactive compounds that contribute to its medicinal properties. This analysis underscores the importance of phytochemical screening in validating traditional medicinal uses and encourages further exploration of its therapeutic potential.

4.2 Effect of drug on body weight

After completion of the experimental protocol, our result indicated that the body weight of diabetic rats significantly decreased as compared to the normal group ($P< 0.05$) as shown in the figure 1. VN treatment at the dose (200 mg/kg, ip) significantly increased the body weight.

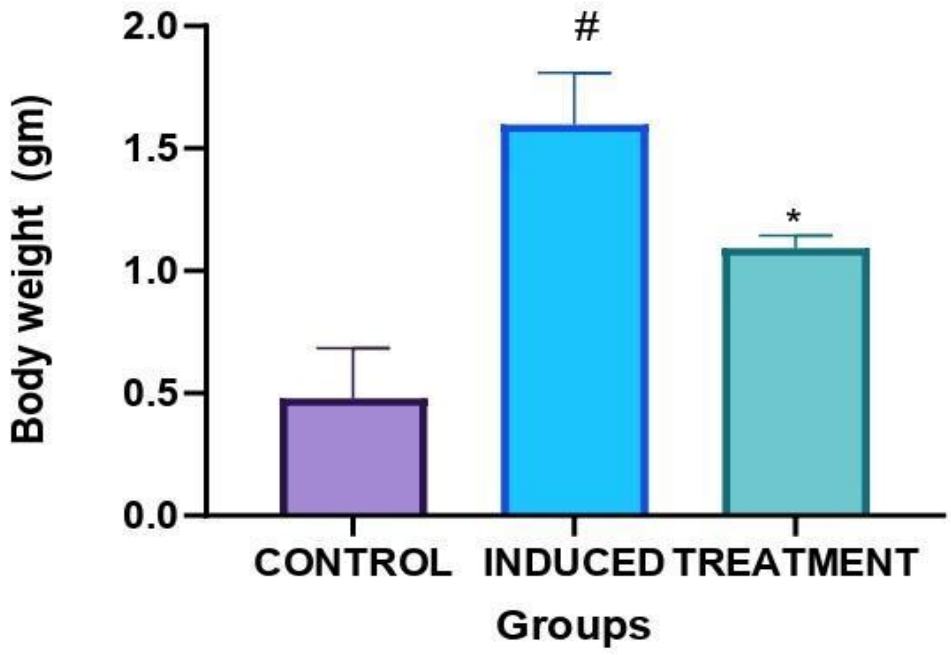

Figure 1: Values are expressed as mean ±SEMs (n=6); #P<0.05, When compared to the normal and *P<0.05, When compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.3 Effect of drug on blood glucose level

After inducing T2DM in animals with STZ and NA, the fasting blood glucose levels of the rats were measured at the end of the study. Compared with the normal group the fasting blood glucose level (FBG) of induced group rats was increased significantly (#P<0.05) (fig 2). Treatment group animals were administered with VN extract, and it was observed that it could reduce the FBG.

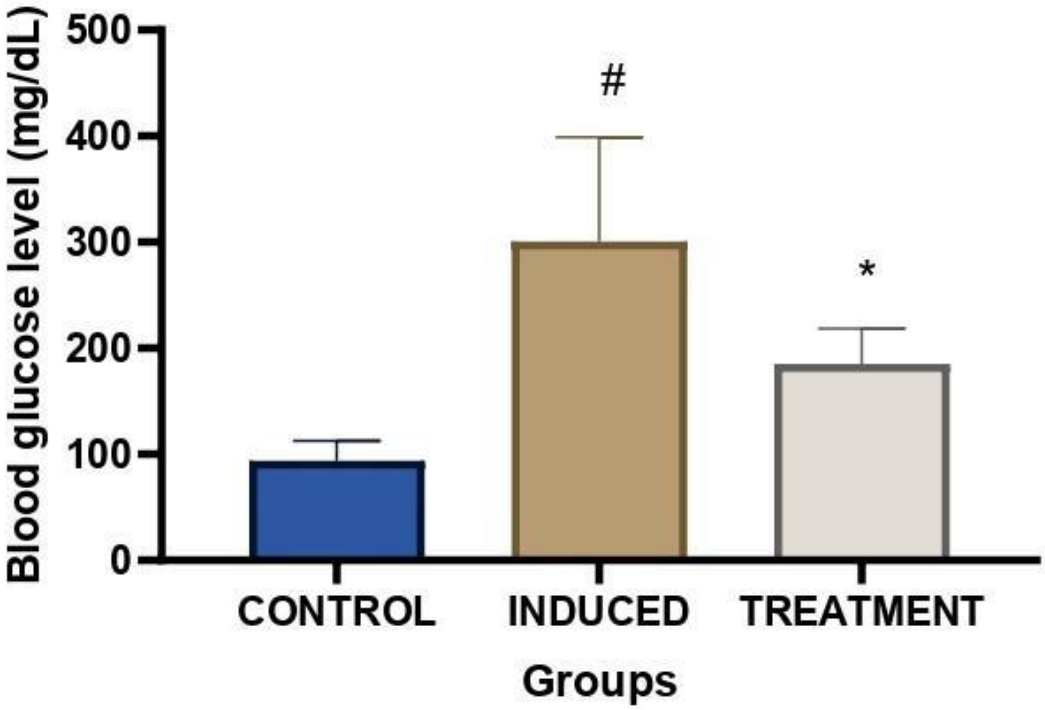

Figure 2: Values are expressed as mean ±SEMs (n=6); # P<0.05, When compared to the normal and *P<0.05, When compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.4 Effect of drug on oral glucose tolerance test

Glucose tolerance was estimated by the oral glucose tolerance test. Compared with the normal group induced rats exhibited a significant elevation (#P<0.05) of FBG at the time points 0, 30, 45, 60 and 120 minutes (fig 3). Our result suggested that the glucose tolerance of diabetic rats was significantly impaired.

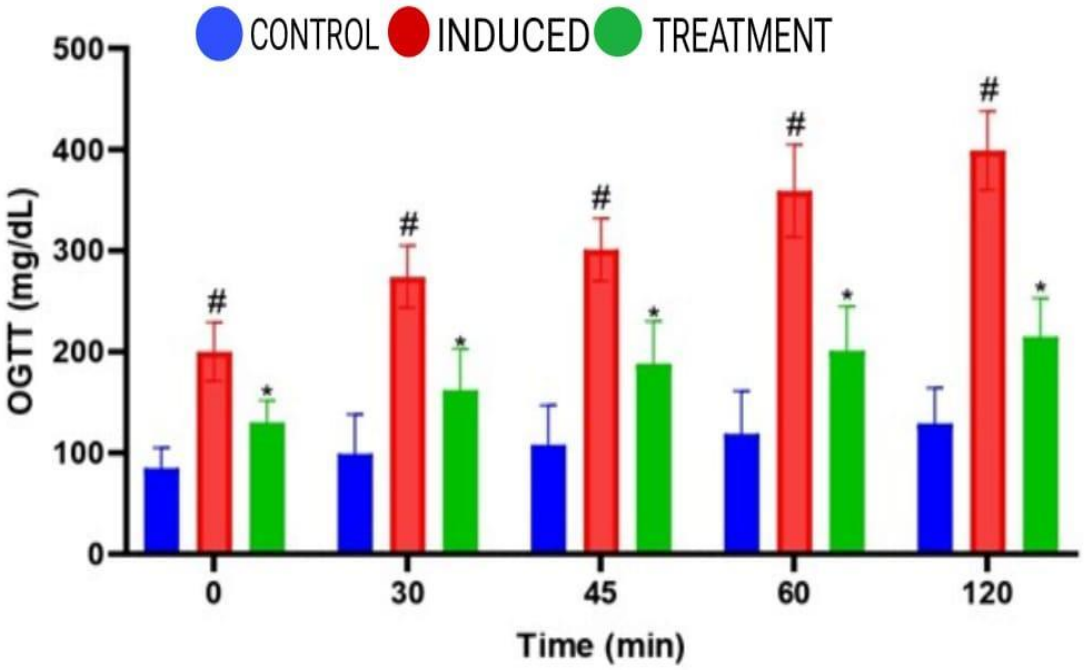

Figure 3: Values are expressed as mean ±SEMs (n=6); # =P<0.05, when compared to the normal and * =P<0.05, When compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.5 Effect of drug on urine albumin

The evaluate effect of VN on STZ-NA-induced diabetic nephropathy; we measured the urine albumin levels. Our findings showed that therapy with VN (200 mg/kg, i.p.) significantly reduced the urine albumin levels in the treatment group compared to induced group (fig 4).

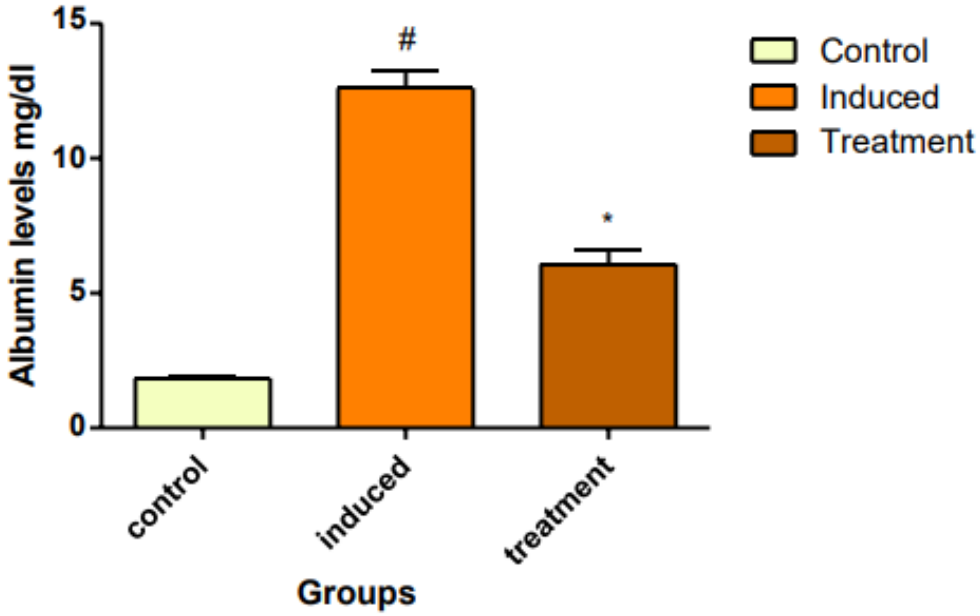

Figure 4: Values are expressed as mean ±SEMs (n=6); #P<0.05, when compared to the normal and *P<0.05, when compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.6 Effect of drug on serum creatinine

We evaluated the effect of VN on STZ-NA-induced diabetic nephropathy, by measuring the serum creatinine levels. Our results pointed out that therapy with VN (200 mg/kg, i.p.) significantly improved the creatinine levels in treatment group rats compared to the induced group whereas the serum creatinine levels in the induced group significantly increased compared to the control group (fig 5).

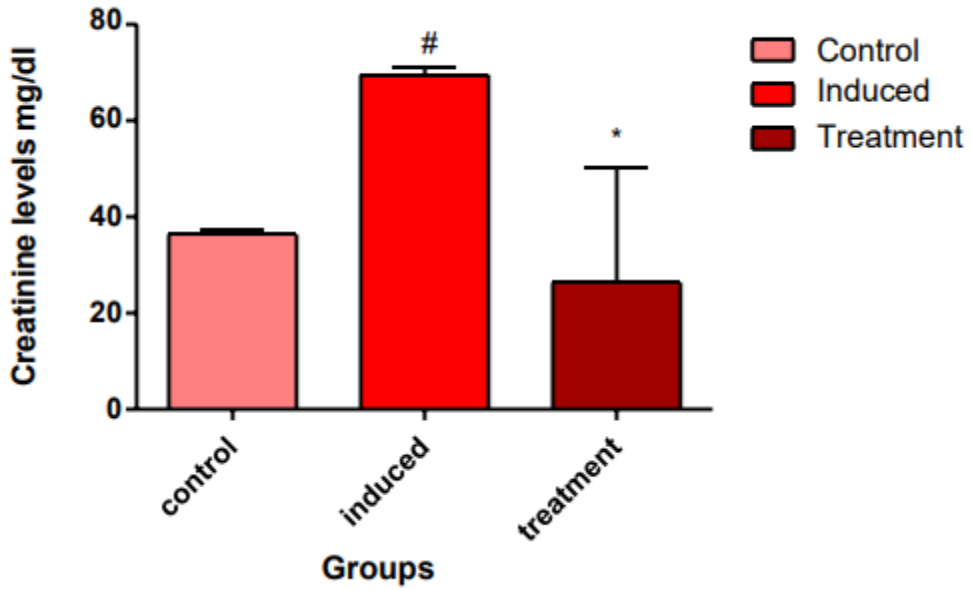

Figure 5: Values are expressed as mean ±SEMs (n=6); #P<0.05, When compared to the normal and *P<0.05, when compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.7 Effect of drug on blood urea nitrogen

To assess the effect of VN on STZ-NA-induced diabetic nephropathy, we measured the BUN levels and evaluated them at the end of the study. Our findings showed that therapy with VN (200 mg/kg, i.p.) significantly improved BUN in the treatment group compared with the induced diabetic group (fig 6).

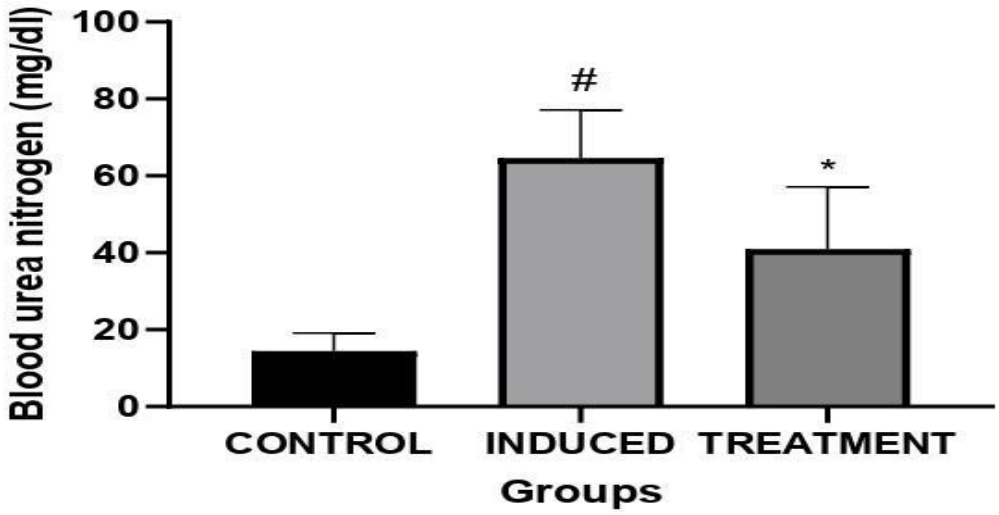

Figure 6: Values are expressed as mean ±SEMs (n=6); #=P<0.05, When compared to the normal and * =P<0.05, When compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.8 Effect of drug on KW/BW ratio

To evaluate the effect of VN in STZ-NA-induced diabetic nephropathy, we measured the KW/BW changes that were checked at the end of the experimental protocol. Our result indicated that treatment with VN (200 mg/kg, i.p) remarkably improved the KW/BW profile in the treatment group compared with the induced group (fig 7).

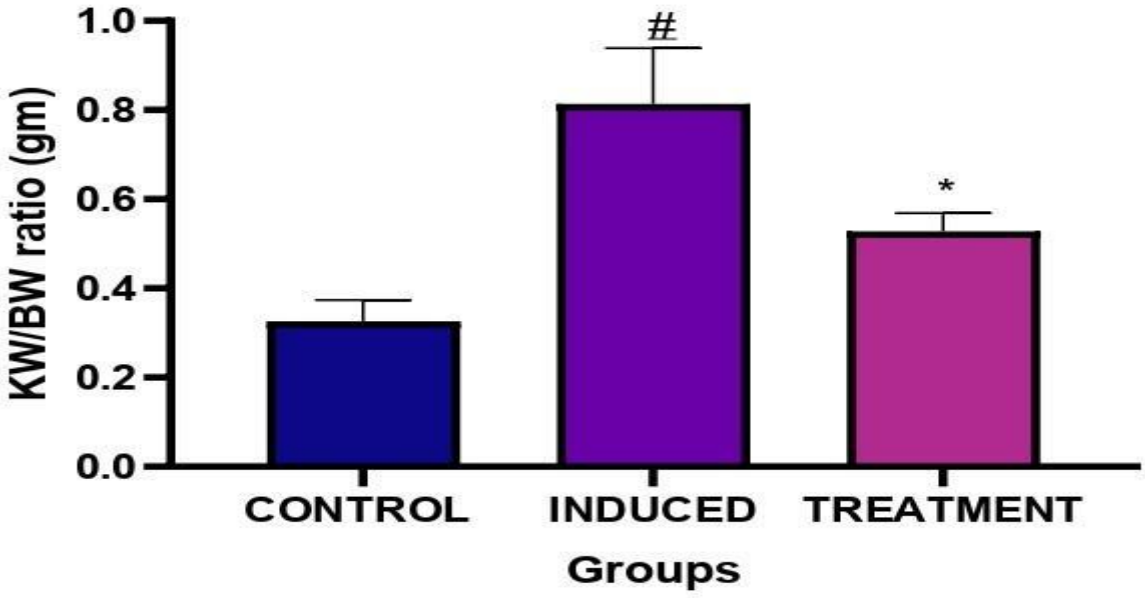

Figure 7: Values are expressed as mean ±SEMs (n=6); #=P<0.05, When compared to the normal and *=P<0.05, when compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.9 Effect of drug on MDA

At the end of the experimental protocol it was observed that ,the MDA levels in the STZ-NA-induced T2DM animals was significantly increased as compared to the normal group (#P<0.05). Moreover, our result also indicated that treatment with VN (200 mg/kg, i.p) remarkably improved the MDA levels in STZ-NA-induced diabetic rats (fig 8).

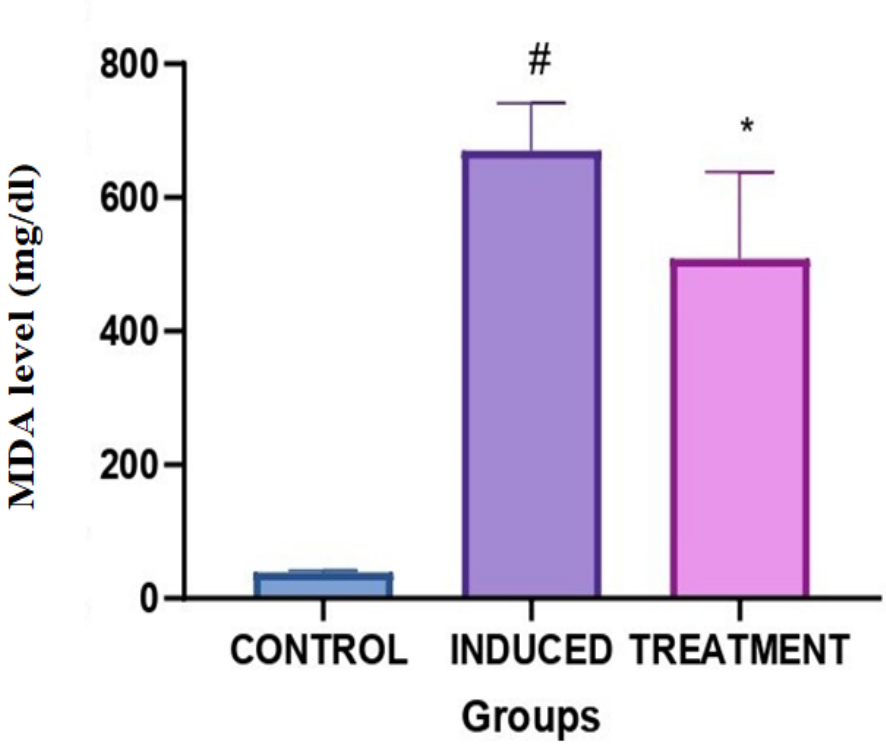

Figure 8: Values are expressed as mean ±SEMs (n=6); #=P<0.05, When compared to the normal and *=P<0.05, When compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group treated with VN extract (200 mg/kg).

4.10 Effect of drug on SOD

SOD levels in the induced T2DM animals was significantly reduced as compared to the normal group (#P<0.05). Moreover, our result indicated that treatment with VN (200 mg/kg, i.p) remarkably improved the SOD levels in the treatment group rats (fig 9).

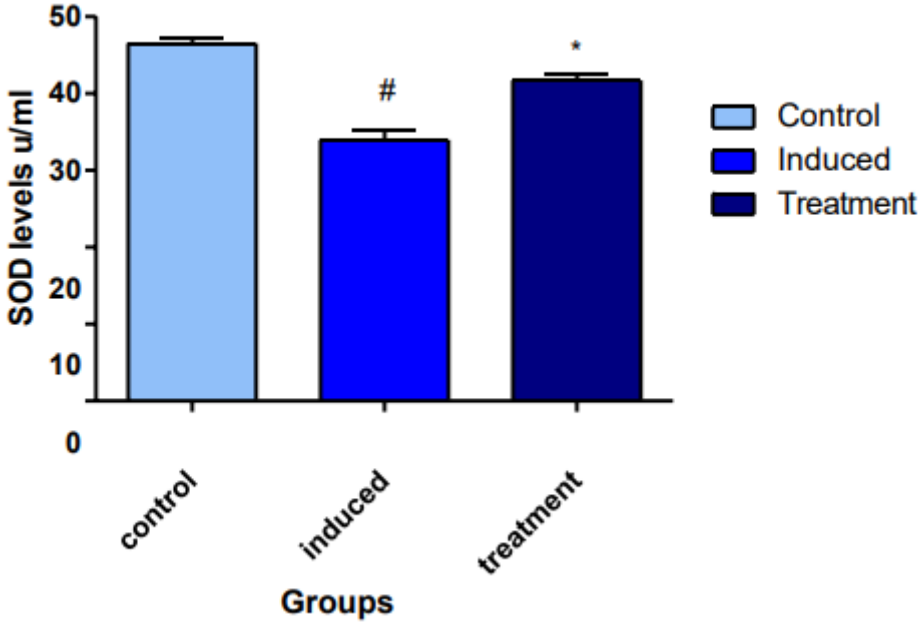

Figure 9: Values are expressed as mean ±SEMs (n=0); #=P<0.05, When compared to the normal and *=P<0.05, when compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group rats treated with VN extract (200 mg/kg).

4.11 Effect of drug on histopathological assessment

Glomerular morphology: Hematoxylin and eosin was used to stain the renal glomeruli of rats in each group. Using Image J software, which is used for image processing, the average diameter of glomeruli was determined. It was discovered that there were more glomeruli in the kidneys of the stimulated rat than in the kidneys of the control rats, indicating a reduction in diameter. Rats treated with rutin had less globally sclerosed glomeruli in their kidneys than rats in the induced group, but more in the kidneys of induced rats than in the control group. The induced group's glomerular diameter was significantly less than that of the control group, while the treated group's glomerular diameter did not decrease relative to the control group.

Tubular morphology: The kidneys of induced rats had higher tubular collapse and thyroidization than the kidneys of control rats. However, in rats treated with the extract, it was significantly lower. The glomerular and tubular changes can be observed in fig 10.

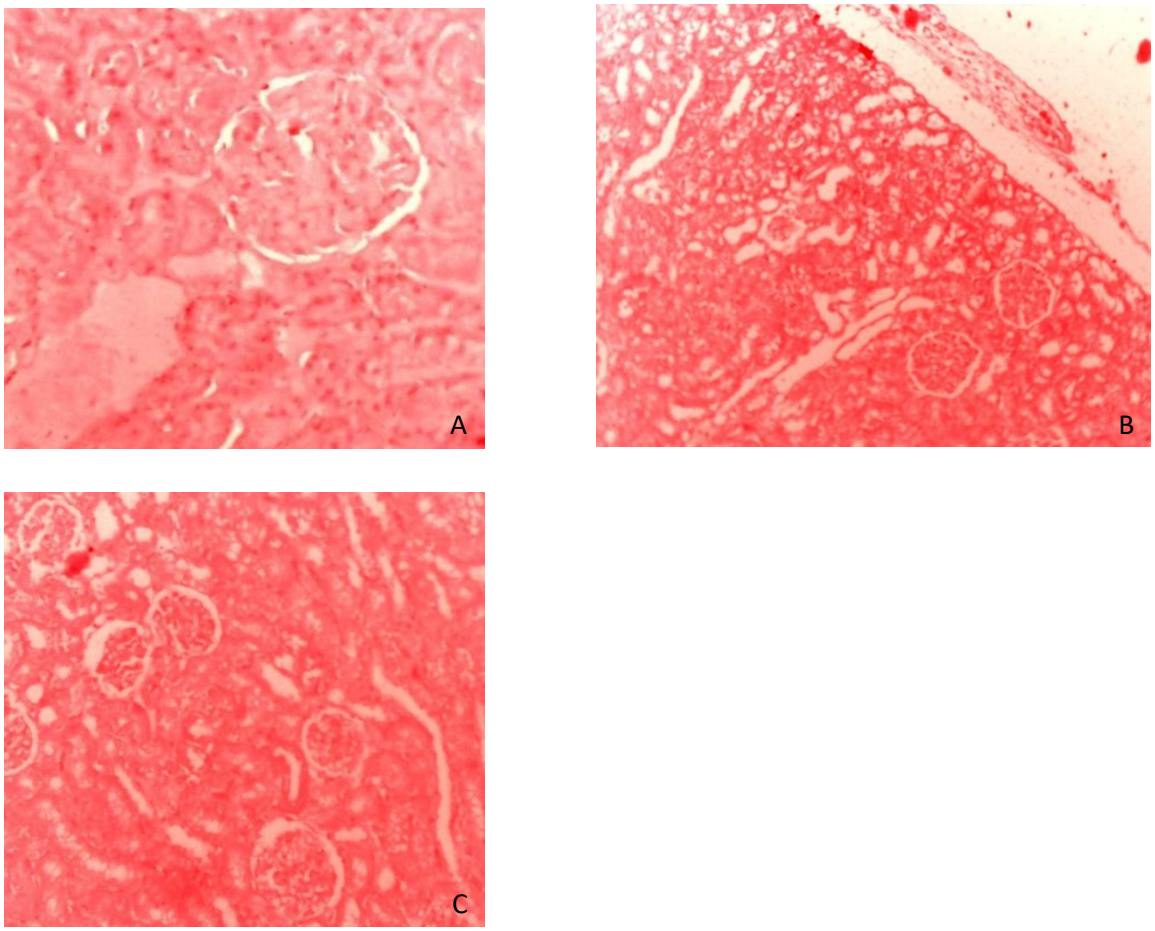

Figure 10: Histopathological changes in kidney tissues in rats of different groups. A. Control B. Induced C. Treatment

4.12 Effect of drug on serum angiotensin levels

Serum Angiotensin II levels in the induced animals was significantly increased as compared to the normal group (#P<0.05). However in the treatment group, serum angiotensin II levels reduced significantly (Fig 11).

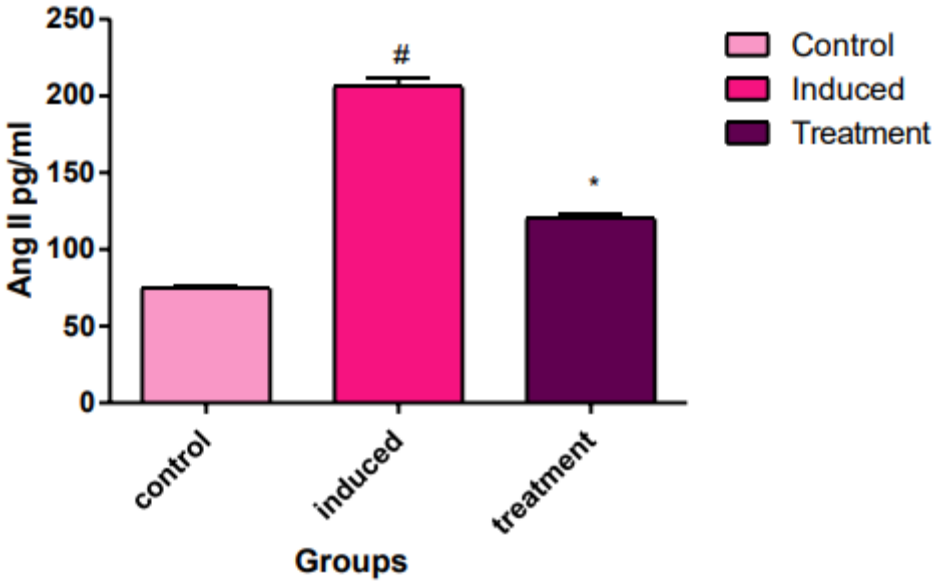

Figure 11: Values are expressed as mean ±SEMs (n=5); #=P<0.05, When compared to the normal and *=P<0.05, when compared to the induced group (one-way ANOVA followed by Bonferroni test). Treatment group rats treated with VN extract (200 mg/kg).

5. Discussion

This study marked the nephroprotective effect of the VN extract in diabetic nephropathy rats. It is well evident from the result that the drug extract has increased the body weight as well as improved the glucose tolerance in rats of the treatment group compared to the induced group. The drug VN extract improved the biochemical parameters related to diabetic nephropathy viz urine albumin, serum creatinine and BUN. The parameters related to oxidative stress like MDA and SOD were also observed to improve with the VN extract. The extract also improved the histopathological parameters like globally sclerosed glomeruli, glomerular collapse and thyroidization of the renal tubules. Lastly the extract also was found to reduce the serum angiotensin II levels compared to the diabetic induced group.

Thus it can be safely assumed that the improvement of the nephropathic parameters as well as the physical and oxidative stress parameters is due to the decrease in the levels of serum angiotensin II. Angiotensin II is directly involved in the inflammatory and the fibrotic pathways in diabetic nephropathy as mentioned in the previous section. Increase in angiotensin II levels can obviously increase the glomerular and tubular injury related to diabetic nephropathy via the NF-Kβ and their related pathways. Thus the decrease in Angiotensin II levels can certainly improve the renal injury by attenuating the NF-Kβ and other related pathways that are coupled with angiotensin II pathways.

While assessment of the chemical constituents, DL Lactamide had the maximum abundance in the extract and along with this Hexitol is another compound which has relatively high abundance. These compounds have the phenolic functional group and may be responsible for the anti-inflammatory and anti-fibrotic activity.

6. Conclusion

This study has highlighted the preventive effect of VN extract on chemically induced diabetic nephropathy via the serum angiotensin II levels. However further studies are yet to be carried out to find out the angiotensin II coupled pathological pathways which can give a more insightful outcome related to pathological prevention of VN extract in diabetic nephropathy.

Funding

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Consent for Publication

I give my consent for publication

Conflict of Interest

There is no conflict of interest between any of the authors related to this study.

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Data availability

Data will be available on request.

Ethics Approval and Consent to Participate

All the ethical approvals were obtained from the Committee for Control and Supervision of Experiments (CCSEA) of Sanaka Educational Trust's Group of Institutions bearing the approval number SETGOI/01/A/2024.

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