Phyllanthus niruri leaves aqueous extract improves kidney functions, ameliorates kidney oxidative stress, inflammation, fibrosis and apoptosis and enhances kidney cell proliferation in adult male rats with diabetes mellitus

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1. Introduction

Diabetic nephropathy (DN) is one of the serious complications of DM (Tziomalos and Athyros, 2015). In DN, kidney damage is histologically characterized by thickening of the glomerular basement membrane, mesangial matrix expansion, macrophage infiltration, loss of podocytes and degeneration of tubular epithelium (Roshan and Stanton, 2013). Recently, attention has been given to oxidative stress as the main factor contributing to DN (Arora and Singh, 2014). Both increased in the production of pro-oxidants and decreased in the action of antioxidants have been reported to be involved in the pathogenesis of DN (Bondeva and Wolf, 2014). In DN, hyperglycemia was reported to trigger oxidative stress, inflammation, fibrosis and apoptosis in the kidney (Xu et al., 2005). Hyperglycemia was also found to enhance formation of advanced glycation end (AGE) and its receptor, RAGE (Singh et al., 2014). In DN, glomerular damage results in protein leakage into the urine which is associated with elevation of blood urea and creatinine levels. Progression of DN ultimately results in end-stage renal failure (ESRF) (Hadjadj et al., 2016).

Phyllanthus niruri is herb belonging to Euphorbiaceae family and is widely used in traditional medicine among the people of the South and Southeast Asia. This plant has been used in the treatment of bronchitis, anemia, leprosy and asthma (Karuna et al., 2009). P. niruri has also been used in Chinese traditional medicine to treat liver injury (Wang, 2000). This herb is found to display hepatoprotective, hypolipidaemic, anti-inflammatory and antihyperuricaemic effects (Lee et al.,...
2016). Recently, *P. niruri* has been found to exhibit anti-diabetic effects (Mediani et al., 2016). It is found to possess antioxidant activities in-vivo and in-vitro (Giribabu et al., 2014). *P. niruri* is widely used in the treatment of urinary tract calculi in different parts of the world. In Brazil, this plant is known as ‘Chanca Piedra’ or ‘stone breaker’ and is considered as a remedy for renal and ureteric calculi (Calixto et al., 1998). In Indian Ayurvedic medicine, *P. niruri* is used to treat renal calculi (Narendra et al., 2012). In Malaysia, *P. niruri*, locally known as ‘dukon anak’ is used to treat kidney disorders including kidney and ureteric calculi (Burkill et al., 1966). Evidences indicate that this plant protects the kidney against stone formation via inhibiting growth of urate crystals (Freitas et al., 2002). Besides, *P. niruri* is also found able to modify composition of kidney stone (Barros et al., 2003) via making it more fragile and easily dissolved (Barros et al., 2006). *P. niruri* is also reported able to induce urethral relaxation as well as reduces excretion of crystallization promoter such as calcium in the urine (Boim et al., 2010). This herb is also proven to be effective in the treatment of hemorrhagic cystitis (Boeira et al., 2011).

Despite of this herb being used in the treatment of stone-related urinary tract disorders, currently there is lack of evidence on its role in ameliorating kidney disease in DM. The notion that *P. niruri* could help to reduce DN progression was supported by an observation that other *Phyllanthus* species i.e. *P. amarus* was found to protect the kidneys against damage due to acetaminophen-induced toxicity in rats (Adeneye and Benebo, 2008b). Therefore, in this study, it was hypothesized that PN was able to protect the kidney against DM-induced nephropathy. This study was aimed to investigate effects of PN on kidney function, kidney histopathological changes and kidney oxidative, inflammatory, fibrosis and apoptosis in DM. Ability of PN to induce kidney cell proliferation in DM was also identified. The leaves was used in view that it is the part of the plant that is most commonly consumed for cooking, as a decoction and as a mixture with beverages example tea (Bagalkotkar et al., 2006; Lee et al., 2016).

2. Materials and methods

2.1. Plant and extraction

The fresh leaves of *P. niruri* were collected from Visakhapatnam, Andhra Pradesh, India. Taxonomic identification was made by botanist and the plant was deposited in a herbarium. The leaves were washed under running tap water and air dried under shade. Dried leaves (800 g) were steeped in cold sterile distilled water (1 L) for three days and aqueous extract was filtered by using No. 1 Whatmann Millipore filter paper (0.45 µm Ref HAWP04700, Bedford, MA, USA). The extract was lyophilized by using a freeze-drying system (Telstar, Barcelona, Spain), yielding 4.46% (w/w) freeze-dried material, which was stored in a cool, dry place until use.

2.2. GC-MS

Identification of the bioactive compounds in PN was performed by using a Trace GC ultra-gas chromatograph, coupled to Quantum XLS mass spectrometer (Thermo Scientific, FL, USA). Compounds were separated by using TG-5MS capillary column (5% phenyl and 95% methyl polysiloxane, 30 m × 0.25 mm i.d. × 0.25 µm film thickness). 1 µl PN was injected into CT splitless mode at injector temperature of 260 °C. GC oven temperature was set at 110 °C, increased by 10 °C/min to 200 °C, then 5 °C/min to 280 °C, ending with 9 min isothermal, at 280 °C. Carrier gas (Helium) flow was at 1 ml/min. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0–2 min and total GC-MS running time was 48 min. Identification was done by comparing the mass spectra with NIST library. The name, molecular weight and structure of the components in the tested materials were then identified.

2.3. Animals

Healthy adult male Wistar rats weighing 180–220 g were procured from National Institute of Nutrition, Hyderabad, India. Animals were maintained at room temperature of 25 ± 2 °C, 12/12 h light/dark cycle. Animals were given standard commercial rat chow diet (Godrej Agrovet, Mumbai, India) and tap water ad libitum. Experimental procedures were in accordance with ARRIVE guidelines (Animals in Research: Reporting In-Vivo Experiments) and European Community Guidelines/ EEC Directive, 1986. All experimental protocols were approved by Institutional Animal Care and Use Committee, Andhra University, with ethics number: AU-IAC/12/2016/009. Acute toxicity study was conducted according to Organization For Economic Cooperation and Development (OECD) revised up-and-down procedure for acute toxicity testing (OECD 425) (OECD, 2001). No signs of toxicity were observed in the tested animals up to dose of 2000 mg/kg/day.

2.4. Induction of DM in experimental animals

After 12 h of fasting, animals were given single injection of nicotinamide (NA) (dissolved in normal saline), intraperitoneally (i.p.), at dose of 110 mg/kg, 15 min later, streptozotocin (STZ) (Sigma, St. Louis, MO) at 55 mg/kg (in 0.1 M sodium citrate buffer, pH 4.5) was also injected i.p. (Giribabu et al., 2016). STZ-NA injected animals were given 5% glucose for 24 h to prevent drug-induced hypoglycemia. Control rats were injected with similar volume of citrate buffer. After 72 h, animals with FBG levels above 300 mg/dl were chosen for the experiment. Treatment was commenced on the fourth day after STZ-NA injection which was considered as day 1. PN was administered at a dose of 200 and 400 mg/kg/day orally in a form of suspension in 1% sodium carboxy methyl cellulose (Na-CMC) in distilled water by using oral gavage tube daily for 28 days. The dosage selection of PN was based on the previous reported doses (Adeneye and Benebo, 2008a; Giribabu et al., 2014; Okoli et al., 2010).

2.5. Experimental Designs

Experimental animals were divided into five (5) groups, each group consist of six rats, as below:

- **Group I:** (NC) Normal control rats - receiving 1% Na-CMC vehicle only.
- **Group II:** (D) Diabetic control rats- receiving 1% Na-CMC vehicle only.
- **Group III:** (D+200PN) Diabetic rats treated with *P. niruri* leaves aqueous extract at 200 mg/kg body weight.
- **Group IV:** (D+400PN) Diabetic rats treated with *P. niruri* leaves aqueous extract at 400 mg/kg body weight.
- **Group V:** (G) Diabetic rats treated with glibenclamide (standard) at 600 µg/kg body weight (Adam et al., 2016b).

2.6. Assessment of body weight and FBG levels

Body weight at day 0 and day 28th. was determined by using a digital weight balance (Sartorius Balance, AX224, Fisher-Scientific, UK). At the end of the experimental period (day 28), animals were placed individually in metabolic cages for 12 h. Urine was collected to measure biochemical parameters. Then blood was collected from retro-orbital plexus and FBG was measured by using commercially available kits (Span Tulip, Goa, India). Measurement was based on glucose oxidase (GOD) and peroxidase (POD) methods, using commercially available kits (Span diagnostic Ltd., Surat, India). At the end of the treatment, rats were anesthetized by i.p. injection of pentobarbitone sodium (60 mg/kg) and sacrificed by cervical dislocation. Kidneys were dissected out immediately and weighted on an electronic balance. Tissue somatic index was determined by using this formula:
Weight of kidney in grams/weight of body in grams x 100.

Blood was collected via direct heart puncture, directly into centrifuge tubes and serum was separated via centrifugation (Thermo Scientific, Model 75005286, USA), at 2000×g for 15 min. Serum was used for biochemical analyses or stored at −80 °C for future use.

2.7. Estimation of creatinine (Cr) clearance

Estimation of serum and urine Cr was made by alkaline picrate method using a commercially available kit (Span diagnostic Ltd., Surat, India). Cr clearance was calculated by using this formula:

\[
\text{Cr clearance} = \frac{\text{urinary Cr(mg/dl) × urine volume(ml)}}{\text{serum Cr(mg/dl) × [1000]} \times \frac{[\text{body weight(g) × [1440(min)]}]}{[\text{1}]}}
\]

Cr clearance was expressed as ml/min/kg body weight.

2.8. Estimation of BUN, BUN/Cr ratio, urea and uric acid levels

Urea and uric acid levels in serum were estimated by using commercially available kits (Span diagnostic Ltd., Surat, India). The values were expressed as milligrams per deciliter of serum.

Cr clearance was obtained by using the following formula:

\[
\text{Urea [mmol/L] = BUN [mg/dL] × 10 [dL/L] /14×2 [mg N/mmol urea]}
\]

2.9. Estimation of urine protein, sodium (Na⁺) and potassium (K⁺) concentrations

Urine protein concentrations were determined by using bicinchninic acid (BCA) assay (Thermo Scientific, Rockford, IL) with BSA used as standard and the results were expressed in mg/dl. Na⁺ and K⁺ levels in the serum were estimated by using commercially available kits (Bio Systems S.A. Costa Brava 30, Barcelona, Spain) and values were expressed as mEq/l.

2.10. Preparation of kidney cytosolic extracts

Excised kidneys were rinsed in ice-cold normal saline. Kidney was minced and homogenized in ice-cold, 0.25 M sucrose solution by using tissue homogenizer, with Teflon pestle (Heidolph Silent Crusher M, Germany), at 4 °C to give 20% homogenate (w/v). Homogenates were then centrifuged (10 min at 8500×g, 4 °C) and supernatant was stored at −80 °C for biochemical and molecular analyses.

2.11. Estimation of lipid peroxidation

Lipid peroxidation in kidney homogenate was measured by estimating the amount of thiobarbituric acid reactive substances (TBARS), by using a commercial kit (Cayman Chemical Item Number 10009055).

2.12. Measurement of TNF-α and IL-6 levels in kidney homogenates by ELISA

TNF-α and IL-6 levels in kidney homogenates were measured by using ELISA kit (Cayman chemicals, Ann Arbor, MI, USA), according to the manufacturer’s guidelines. Cytokines levels were determined from a standard curve and were expressed in ng/mg protein.

2.13. Nuclear extraction and NF-kB p65 activity measurement in kidney homogenates

Nuclear extractions were prepared from kidney homogenates by using nuclear extract kit (Cat#40010, Active Motif, Carlsbad, CA, USA), following the manufacturer’s protocol. NFkB p65 DNA binding activity was determined by using TransAM® NFkB p65 protein assay kit (#Cat No. 40096, Active Motif, Carlsbad, CA, USA) following the method as previously described (Aziz et al., 2017). The results were expressed as NF-kb p65 concentration per unit of optical density.

2.14. Histopathological studies

Kidneys were histologically fixed in 10% buffered formalin following harvesting, then embedded in paraffin and manually cut into 5 μm-thick sections by using a microtome (Histo-line laboratories, ARM-3600, Viabrembo, Milan, Italy). Sections were then dewax in two changes of xylene, hydrated in two changes of 100% ethanol, followed by 95% and 80% ethanol and finally rinsed with H₂O. Sections were then stained with hematoxylin and eosin (H&E). The stained sections were dehydrated with 80% ethanol followed by 95% ethanol, placed in two changes of 100% ethanol and cleansed with two changes of xylene. Histopathological changes were viewed by using a phase contrast microscope (Nikon H600L, Nikon DS camera control Unit DS-U2, Version 4.4), with an attached photograph machine (Nikon H600L, Japan).

2.15. Morphometric analysis

Morphometric analysis of the renal corpuscle and glomeruli were performed by using Image J software (Image J 1.39f, NIH-Bethesda, MD, USA). From each group, six histological sections were examined at different magnifications to obtain average (1) diameter of renal corpuscles, (2) diameter of glomerulus, (3) glomerular cross sectional area and (4) diameter of Bowman’s capsule from 50 corpuscles under low power field (10×). Mean glomerular volume (VG) was calculated as follows:

\[
\text{VG = area}^3 \times \text{1.38} \times \text{1.01}
\]

\[
\text{VG=1.5×1.38/1.01, where 1.38 represents the shape coefficient, and 1.01 represents size distribution coefficient.}
\]

2.16. Distribution of protein by immunoperoxidase and immunofluorescence

For immunohistochemistry, sections were deparaffinized by immersing in xylene for 20 min, and then dropped in ethanol at decreasing concentrations as above, 5 min each. Antigen retrieval was performed by incubating the sections in 0.01 M citrate buffer, pH 6.0 for 10 min at 100 °C. Subsequently 3% H₂O₂ in PBS was used to neutralize the endogenous peroxidase. Sections were blocked in 5% BSA for non-specific binding, prior to incubation with RAGE, Nr2f, TNF-α, IL-6, Casp-3, TGF-β1, FGF-1, Ki-67 primary polyclonal antibodies (sc-8230; sc-722; sc-1351; sc-1266; sc-1225; sc-1884; and sc-15402; Santa Cruz, CA, USA respectively), at a dilution of 1:50 in 5% normal serum (Santa Cruz, CA, USA) prior to incubation with Ikk-β, IL-1β, Casp-9, Bax and VEGF polyclonal primary antibodies (sc-34674; sc-7884; sc-7885; sc-526; sc-507; Santa Cruz, CA, USA), at a dilution of 1:100 in PBS with 1.5% normal blocking serum at room temperature.
for 1 h. After three times rinsing with PBS, sections were incubated with IgG–fluorochrome-conjugated secondary antibody (Santa Cruz, CA, USA), at a dilution of 1:250 in PBS with 1.5% normal blocking serum at room temperature for 45 min. The slides were rinsed three times with PBS and were mounted with Ultracruz mounting medium (Santa Cruz, CA, USA), counterstained with DAPI to visualize the nuclei.

2.17. mRNA quantification by Real-time PCR (qPCR)

Kidney were kept in RNA Later solution (Ambion, Austin, TX, USA) prior to RNA extraction. Tissues were homogenized in liquid nitrogen using mortar and pestle. Total RNA was freshly isolated by using mortar and pestle Total RNA was freshly isolated by using PRO-PREP solution (INtRON Technologies. N. Giribabu et al.) and synthesized by Integrated DNA meres were designed by NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Integrated DNA Technologies.

Two steps Real-time PCR was used to evaluate gene expression. Reverse transcription into cDNA was performed by using high capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Target genes were amplified using standard real-time PCR kit (Applied Biosystems, Foster City, CA, USA). In real-time PCR, amplification was performed using QuantiNova SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) on Applied Biosystems StepOne Plus Real-Time PCR Systems. RNA fold changes were calculated according to comparative Ct (2−ΔΔCt) method. Primers used for amplification of the respective gene used in this study were as follows: RAGE (Ager) (5′-AGG TGT CAC AG GAA AAC CCG GTG TAT-3′ and 5′-GG CCT CCCAG GAT CCA TTCA-3′), IKK-β (5′-CCT GTG CGT GAG TAG CTA A-3′ and 5′-GTG CC GT TAA GCT CTC TTGA-3′), Bax (5′-CACA AGA AGG CTG CAG GT-3′ and 5′-CACA TAGA CAC TAG CTC TCT-3′), VEGF (5′-GAC ACC ATC GTG AAC AGC ATC-3′ and 5′-GAC CCT GAG CTT GGC ATAC-3′), TGF-β1 (5′-CTG TCT GAC CCC CAC TCT A-3′ and 5′-AGC CTT AT TCC GCT TC-3′), FGF-2 (5′-TTC TCT CCG CAT CTC CAC C-3′ and 5′-GCTG TAG TGG TGC GGT GGG-3′), PCNA (5′-GA AGG CTT GA GAA CAC CCG T-3′ and 5′-TTT TGG CAT GC TGG GAG GT-3′), Gapdh (5′-GCT TCT TTC TTG CAG GTG GGC C-3′ and 5′-TAAG GCC AAAT CCG TCA C-3′). The primers were designed by NCBI Primer-Blaster tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Integrated DNA Technologies.

2.18. Western blotting

Peri-renal fats were removed and kidneys were rinsed with 0.1% phosphate buffered solution. Tissues were then snapped frozen in liquid nitrogen and stored at −80 °C prior to protein extraction. Total amount of protein was extracted from 40 mg (wet weight) tissue. After extraction of total protein with PRO-PREP solution (INNOR Biotechnology, South Korea), equal amount of protein from each tissue lysate were mixed with loading dye, boiled for 5 min and separated by SDS-PAGE 10%. Protein was then transferred onto PVDF membrane (BIO-RAD, Hercules, CA, USA) and blocked with 5% BSA for 90 min at room temperature. The membranes were then exposed to goat polyclonal primary polyclonal SOD-1 (sc-271014), CAT (sc-50508), GPx-1 (sc-22145), IL-1β (sc-7884), Casp-3 (sc-1225), Casp-9 (sc-7885), PCNA (sc-9857), GADPH (sc-25778), Vinculin (sc-5573) (Santa Cruz, CA, USA) diluted at 1:1000 in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and Tween 20 for 90 min. Blots were washed 3 times, 5 min each, and finally incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotech Inc, CA, USA) at a dilution of 1:2000, for 1 h. The membranes were washed and subjected to Opti-4CN™ Substrate Kit (Bio-Rad, USA) to visualize the protein bands. Density of each band was determined by using Image J software (1.39, NIH-Bethesda, MD, USA). The ratio of each target band/GAPDH or vinculin was calculated and was considered as the expression level of the target proteins.

2.19. Statistical analysis

Student’s t-test was used to compare between different samples. p < 0.05 was considered as significant. Results were expressed as mean ± standard deviation (S.D.). Post-hoc statistical power analysis was performed and values obtained were > 0.8 which indicate adequate sample size.

3. Results

3.1. Effects of PN on body weight and FBG levels

The body weight of diabetic rats declined steadily following STZ injection (Table 1). In PN or glibenclamide-treated diabetic rats, body weight at 0th day 195.34 ± 8.55 212.54 ± 11.24 216.25 ± 9.42 205.55 ± 8.53 208.36 ± 11.46
Body weight at 28th day 210.42 ± 9.46 157.47 ± 10.45 176.34 ± 9.63 195.65 ± 9.32 187.46 ± 11.83
Blood glucose levels at 0th day (mg/dl) 88.75 ± 9.5 358.64 ± 14.53 347.56 ± 16.79 349.65 ± 13.25 354.68 ± 18.46
Blood glucose levels at 28th day (mg/dl) 90.68 ± 9.21 346.43 ± 15.53 208.75 ± 15.85 152.37 ± 17.43 146.28 ± 12.59
Kidney tissue somatic index (%) 0.64 ± 0.07 0.52 ± 0.05 0.55 ± 0.06 0.59 ± 0.08 0.62 ± 0.08
Serum creatinine (mg/dl) 0.62 ± 0.06 1.76 ± 0.08 1.05 ± 0.07 0.87 ± 0.08 0.75 ± 0.06
Urinary creatinine (mg/dl) 57.26 ± 2.89 18.35 ± 1.92 25.68 ± 1.54 32.72 ± 2.36 38.65 ± 2.71
Urinary output (ml) 8.52 ± 0.76 38.72 ± 0.59 26.28 ± 0.48 21.83 ± 0.86 18.97 ± 0.67
Creatinine clearance (ml/min/kg bw) 2.17. mRNA quantification by Real-time PCR (qPCR)
weight was markedly higher than un-treated diabetic rats (p < 0.05). Serum FBG levels in diabetic rats at day 0 was markedly higher than in non-diabetic rats where no significant changes were noted in the former after 28 days. However, treatment of diabetic rats with PN or glibenclamide for 28 days resulted in significant lowering of FBG levels (p < 0.01).

3.2. Effects of PN on kidney functions

In un-treated diabetic rats, weight of the kidneys was significantly higher than non-diabetic rats where no signifi

Fig. 1. (A): Effects of PN on kidney histopathology, (B) high magnification image showing glomerular appearance. (A) Sections of normal, non-diabetic rats kidney showing glomerular and renal tubular histology. Shrink glomeruli could be seen in the kidney section from diabetic rats. Kidney sections from PN and glibenclamide-treated diabetic rats showed less morphological changes with slightly shrink glomeruli (H & E 400×). (B) High number of mesangial cells and low number of podocytes were observed in diabetic rats' kidneys. PN treatment caused low number of mesangial cells and high number of podocytes (1000X). GR- Glomeruli, M - Mesangial cell, P - Podocyte, GCL- Glomerular capillary lumina. Scale bar =50 µm.
decreased however serum Cr, uric acid, BUN, Ccr, Na+, K+, urine output and urine protein were significantly increased as compared to non-diabetic rats (p < 0.05) (Table 1). Treatment of diabetic rats with PN or glibenclamide for 28 days ameliorated reduction of kidney weight while maintaining near normal serum Cr, uric acid, BUN, Ccr, serum Na+, K+, urine output and urine protein levels. In diabetic rats, urine Cr, Ccr and BUN/Cr ratio decreased, and these decrease were ameliorated following treatment with PN or glibenclamide.

3.3. Effects of PN on histopathological changes in the kidney

In non-diabetic rats, kidney appeared normal as featured by normal appearance of proximal and distal convoluted tubules (PCT and DCT), renal corpuscles and glomerulus (G) (Fig. 1A). However, in diabetic rats, kidneys showed abnormal features such as atrophied glomeruli and necrosis of the tubules. These abnormal changes were ameliorated in diabetic rats treated with PN or glibenclamide.

Higher magnification images showed higher number of mesangial cells with lower number of podocytes in diabetic rat glomeruli when compared to non-diabetic rat glomeruli (Fig. 1B). In PN or glibenclamide-treated diabetic rats, lower number of mesangial cells but higher number of podocytes were observed in the kidney when compared to non-treated diabetic rats.

The average diameter of renal corpuscles, glomerulus, Bowman capsules, glomerular cross sectional area and glomerular volume in diabetic rats were significantly lower when compared with non-diabetic rats (p < 0.01) (Table 2). 28 days treatment of diabetic rats with PN or glibenclamide resulted in significantly higher average diameter of renal corpuscles, glomerulus, Bowman capsule, glomerular cross sectional area and glomerular volume (p < 0.01).

3.4. Effects of PN on kidney oxidative stress

In diabetic rats, RAGE protein was abundantly expressed in the tubular regions of the kidney (Fig. 2A). Treatment of diabetic rats with PN or glibenclamide resulted in a relatively lesser RAGE expression in kidney tubules. Significantly higher Rage mRNA levels were noted in diabetic rat kidney as compared to non-diabetic rat kidney (p < 0.05) (Fig. 2B). In diabetic rat kidney, Rage mRNA levels were significantly reduced following PN or glibenclamide treatment (p < 0.05).

In diabetic rats, nuclear factor-erythroid-2-related factor 2 (Nrf2) was found to be distributed in the nuclei of kidney cells (Fig. 2C and D). However, in diabetic rats receiving PN or glibenclamide, even higher Nrf2 nuclei distribution was observed (p < 0.05).

TBARS was found to be significantly increased in the kidney of diabetic rats when compared to kidneys of non-diabetic rats (p < 0.05) (Fig. 2E). Treatment of diabetic rats with PN or glibenclamide resulted in significantly lesser TBARS level in kidneys. SOD-1, CAT and GPx-1 expressions in diabetic rat kidney were significantly decreased when compared to non-diabetic rat kidneys (p < 0.05) (Fig. 2F). Treatment of diabetic rats with PN or glibenclamide resulted in significantly higher SOD-1, CAT and GPx-1 expression in the kidney.

3.5. Effects of PN on kidney inflammation

NFκB p65 DNA binding activity in diabetic rat kidneys was significantly higher as compared to non-diabetic rat kidneys (p < 0.05) (Fig. 3A). Treatment of diabetic rats with PN or glibenclamide resulted in lower NFκB p65 level in the kidney. Ikk-β mRNA levels were significantly higher in the kidney of diabetic rats when compared to non-diabetic rats (p < 0.05) (Fig. 3B). In diabetic rats, treatment with PN or glibenclamide resulted in Ikk-β mRNA levels in the kidney to decrease (p < 0.05). Immunofluorescence staining showed Ikk-β protein was predominantly distributed in the tubular and glomerular regions of the kidneys at a relatively higher levels in diabetic rats than non-diabetic rats (Fig. 3C). Treatment with PN or glibenclamide resulted in Ikk-β levels in the kidney of diabetic rats to reduce.

TNF-α levels were significantly higher in the kidney of diabetic rats as compared to non-diabetic rats (p < 0.05) (Fig. 3D). Diabetic rats treated with PN or glibenclamide had lower TNF-α levels in kidneys (p < 0.05). Immunohistochemistry images indicated that TNF-α were highly distributed in the tubular regions of the kidney of diabetic rats. However, a relatively lower distribution of TNF-α was observed in kidney tubules of diabetic rats following treatment with PN or glibenclamide (Fig. 3E).

Immunohistochemistry images showed IL-6 was highly distributed in the tubules and glomerulus of diabetic rat kidneys (Fig. 4A). Diabetic rats treated with PN or glibenclamide had a relatively lower IL-6 distribution in the kidney tubules and glomeruli.

IL-6 levels in diabetic rat kidneys were significantly higher when compared to non-diabetic rat kidneys (Fig. 4B). In diabetic rats, PN or glibenclamide treatment resulted in IL-6 levels in kidneys to decrease. Higher expression of IL-1β was observed in kidneys of diabetic rats when compared to non-diabetic rats with PN or glibenclamide treatment reversed these changes (Fig. 4C). Immunofluorescence images showed a relatively higher IL-1β distribution in the glomeruli and tubular regions of kidney of diabetic rats (Fig. 4D). Treatment of diabetic rats with PN or glibenclamide resulted in IL-1β distribution in the glomeruli and tubules to decrease.

3.6. Effects of PN on kidney apoptosis

Immunofluorescence images show high distribution of Bax in the glomeruli and tubules of diabetic rat kidneys (Fig. 5A). In diabetic rats receiving PN or glibenclamide treatments, a relatively lower distribution of this protein was observed in the glomeruli and tubules. Bax mRNA levels were highest in diabetic rat kidney and its levels were reduced following treatment with PN or glibenclamide (Fig. 5B). Immunohistochemistry images showed high distribution of caspase-3 in tubules of diabetic rat kidneys with administration of PN or glibenclamide resulted in distribution of this protein to be relatively decreased (Fig. 5C). In the meantime, immunofluorescence images showed high distribution of caspase-9 in the renal corpuscles and tubules of diabetic rat kidneys where treatment with PN or glibenclamide caused distribution of caspase-9 to be relatively decreased (Fig. 5D). Caspase-3 and Caspase-9 protein expression levels were
highest in diabetic rat kidneys (p < 0.05) (Fig. 5E). In diabetic rats, treatment with PN or glibenclamide resulted in caspase-3 and caspase-9 protein expression levels in the kidney to decrease (p < 0.05).

3.7. Effects of PN on kidney fibrosis

Immunofluorescence images showed high distribution of VEGF in the glomerulus and tubules of diabetic rat kidneys (Fig. 6A). Distribution of VEGF in the glomeruli and tubules was markedly decreased when diabetic rats were given either PN or glibenclamide. The levels of Vegf mRNA were highest in the kidney of diabetic rats (Fig. 6B). However, following PN or glibenclamide treatment, Vegf mRNA levels markedly decreased. TGF-β1 was highly expressed in the tubules of diabetic rat kidneys (Fig. 6C). Tgf-β1 mRNA levels were also highest in diabetic rat kidneys (Fig. 6D). In these rats, treatment with PN or glibenclamide resulted in significant reduction in Tgf-β1 mRNA levels. Immunohistochemistry images showed FGF-1 was highly distributed in the tubules of diabetic rat kidneys (Fig. 6E). However, following treatment of diabetic rats with PN or glibenclamide, distribution of FGF-1 in tubules reduced. Fgf mRNA levels were highest in the kidney of diabetic rats and were markedly reduced following PN or glibenclamide treatments (Fig. 6F).

3.8. Effects of PN on kidney proliferation

Immunohistochemistry images showed low distribution of Ki-67 in the kidney of diabetic rats (Fig. 7A and B). Ki-67 could be seen to be localized in the nuclei. In diabetic rats, distribution of Ki-67 was relatively higher following treatment with PN or glibenclamide. Expression levels of PCNA protein in diabetic rat kidneys was
significantly lesser when compared to the kidney of non-diabetic rats (Fig. 7C). In diabetic rats, treatment with PN or glibenclamide resulted in significantly higher PCNA expression in the kidney. Similarly, *Pcna* mRNA levels were lowest in the kidney of diabetic rats. In diabetic rats which received PN or glibenclamide treatments, *Pcna* mRNA levels were significantly increased (Fig. 7D).

3.9. Identification of the compounds in PN by GC-MS

Analyses of GC-MS chromatogram showed the presence of 12 peaks which represent 12 major compounds in the extract (Fig. 8A and B). The retention time (RT), molecular formula and molecular weight (MW) of these compounds are shown in Table 3. The major compounds present are phenol, 4-(2-phenylethyl) (Rt 15.106); tyrosine (Rt 16.880); n-hexadecanoic acid (Rt 33.588); 9,12-octadecadienoic acid (Z,Z) (Rt 36.775); trans-13-octadecenoic acid (Rt 36.907); tetradecane (Rt 43.006); octacosane (Rt 44.540); tetradecane (Rt 46.010); octacosane (Rt 47.441); 6,7-epoxypregnen-4-ene-9,11,18-triol-3,20-dione, 11,18-diacetate (Rt 48.688).

4. Discussion

This study has shown that administration of PN to diabetic rats was able to prevent deterioration of kidney functions via ameliorating...
histopathological changes in the kidney particularly in the renal corpuscles and tubules. These effects could be due to the reduced levels of oxidative stress, inflammation, fibrosis and apoptosis. Diabetic rats was found to have elevated FBG levels which was significantly ameliorated following treatment with PN, in a dose-dependent manner. The anti-hyperglycemic effects of *P. niruri* was also reported in alloxan-induced diabetic rats (Okoli et al., 2011). Our findings indicated that treatment of diabetic rats with PN was able to ameliorate the decreased in kidney somatic index which could be due to decrease in protein and/or glycogen degradation.

Administration of PN to diabetic rats was found able to improve therenal function as indicated by decreased in the urine output, most probably due to near normal FBG levels. Ability of PN to ameliorate increased in plasma electrolytes i.e. Na⁺ and K⁺ levels in diabetic condition could be due to its ability to decrease the urine output, thus ameliorating dehydration. In the meantime, near normal K⁺ levels in diabetic rats as observed following PN treatment could be due to ability of the plant extract to maintain near normal plasma insulin level that is essential for the regulation of plasma K⁺ (Zierler et al., 1966).

Administration of PN to diabetic rats was found able to decrease the serum uric acid levels, but the underlying mechanisms are unknown. There has been a report which indicate that *P. niruri* leaves methanolic extract was able to lower the serum uric acid levels in hyperuricemic rats (Murugaiyah and Chan, 2006). An increased in serum Cr and blood urea levels in diabetic rats indicated the decreased renal function, the findings consistent with others (Perrone et al., 1992). In the meantime, elevated urea levels indicated the possibility of renal failure (Nissenson, 1998) while decreased in BUN/Cr ratio indicated that the failure most likely due to intrinsic damage to the kidney (Irwin and Rippe, 2008; Lavizzo-Mourey et al., 1988). Preservation of near normal serum Cr, urea, BUN and decreased in BUN/Cr ratio suggested that PN was able to preserve near normal kidney functions in DM.
Deterioration of kidney functions as observed in diabetic rats was consistent with the histopathological changes in the kidneys as featured by glomerular atrophy and reduced surface area of the Bowman capsule. These changes would likely contribute towards decreased in GFR. Administration of PN to diabetic rats reduced the loss of podocytes, in which decreased in podocytes is a characteristic of DN (Steffes et al., 1992). In DM, podocyte loss is associated with glomerulosclerosis (GS) (Sayyed et al., 2009). In this study, reduced number of renal corpuscles in diabetic rat kidneys indicated renal atrophy, which would result in decreased kidney ultrafiltration (Jones et al., 1979). Additionally, variations of the size of renal corpuscles in diabetic rats indicate compensatory hypertrophy which could help to sustain the normal GFR. Following administration of PN, reduced inter-glomeruli size variations and increased in glomerular diameter, glomerular cross-sectional area and glomerular volume in diabetic rats indicate amelioration of renal atrophy following administration of the extract. Additionally, reduced urine protein concentration following PN administration indicated reduced glomerular basement membrane damage, the features that characterized DM-induced intrinsic kidney disease (Lahdenkari et al., 2004).

In this study, ability of PN to partially alleviate deterioration of renal function could be due to amelioration of oxidative stress, inflammation, apoptosis and fibrosis in the kidney and improvement of the kidney cell proliferation. The increased in oxidative stress in diabetic rat kidney was evidenced from increased levels of RAGE and lipid peroxidation (LPO), the latter is a reflection of tissue oxidative damage in addition to decreased in SOD, CAT and GPx anti-oxidative enzymes. Administration of PN ameliorated DM-induced oxidative stress. In addition, in DN, tissue damage was also due to increase in peroxide and NO free radicals levels (Onozato et al., 2002).

![Fig. 5](image-url)
In DM, levels of Nrf2, a well-known cytoprotective factor were found to increase, where this factor could help to protect the kidney via orchestrating the antioxidant responses to oxidative stress. Elevated ROS levels prevent degradation of Nrf2 and activate the formed Nrf2 to translocate into the nucleus, after which it activates the antioxidant response elements (AREs)-regulated genes, leading to downstream transcription of antioxidant enzymes (Kim and Vaziri, 2010; Ruiz et al., 2013). PN administration to diabetic rats increased Nrf2 expression where this could help to up-regulate ARE-regulated genes causing increased in antioxidant enzymes’ levels. Lack of increased in Nrf2 in non-diabetic rats was expected as there were no pathology that triggers increased in Nrf2 levels. Our findings were consistent with previous studies which indicate that natural products activate Nrf2 as well as scavenges free radicals via activating its downstream targets in many disease conditions (Xu et al., 2016; Li et al., 2016; Pandurangan et al., 2015; Park et al., 2016). Increased in Nrf2 expression in diabetic rats following PN administration would increase cytoprotection and this was supported by studies which reported that aqueous and methanolic extracts of *P. niruri* leaves displayed in-vitro free radical scavenging activities (Harish and Shivandanappa, 2006; Sabir and Rocha, 2008). Besides, *P. niruri* leaves extracts were also found to prevent toxins (Manjrekar et al., 2008) and drug-induced oxidative stress in the liver in rats (Chatterjee and Sil, 2006; Sarkar and Sil, 2007).

In this study it was found that the levels of inflammation were high in diabetic rat kidney as indicated by increased levels of NF-kB, Ikkβ, TNF-α and ILs (IL-1β and IL-6). These findings were consistent with a report which indicate that TNF-α levels in the kidney were markedly

![Image of fluorescence images showing distribution of VEGF, Vegf mRNA levels, TGF-β, FGF-1, and Tgf-β mRNA levels](attachment:image6.jpg)
increased in DM (McCarthy et al., 1998). Inflammation was found to be triggered by hyperglycemia and oxidative stress (Adam et al., 2016a; Ojha et al., 2014). Activation of Nrf-2 as well as translocation of Nrf2 into the nucleus due to increase in LPO and free radicals levels could trigger NF-κβ activation, inducing transcription of genes that encodes the inflammatory proteins (Roslan et al., 2017; Zhong et al., 2016). Ability of PN to reduce inflammation in the kidney could either be due to direct or indirect effects via decreased in oxidative stress and hyperglycemia.

In this study, it was found that fibrosis levels in the kidney were markedly increased in DM and were ameliorated following PN administration as reflected by decreased in TGF-β1, VEGF and FGF1 levels. Previous reports indicated that DN is associated with high fibrosis levels (Ha and Kim, 1999). High glucose levels were able to stimulate synthesis of collagen and matrix glycoproteins in the mesangial cells and interfered with the degradation of glycosylated proteins (Abrass, 1995). Meanwhile, increased in TGF-β1 expression could result in mesangial matrix expansion (Kagami et al., 1994) and decreased in podocytes number in DM (Wolf and Ziyadeh, 2007). Decreased in the population of mesangial cells in diabetic rat kidneys following PN administration could be due to amelioration in fibrosis.

The levels of apoptosis in kidneys which were high in DM were markedly reduced following PN administration as reflected by reduced levels of Bax, caspase-3 and caspase-9. In the meantime, reduced cell proliferation in the kidneys in DM as reflected by reduced levels of PCNA and Ki-67 were ameliorated following PN administration. Levels of apoptosis were reportedly high in DM (Verzola et al., 2007). Hyperglycemia, inflammation and oxidative stress could trigger increased in Bax levels, which activates the apoptosis signaling pathway encompassing of caspase-3 and caspase-9 (Song et al., 2014).

Some of the bioactive compounds in PN could account for these observed biological effects. n-Hexadecanoic acid (palmitic acid) has been shown to exhibit antioxidant (Sutha et al., 2012) and anti-inflammatory effects via inhibition of phospholipase A(2), an enzyme that is involved in controlling inflammation (Aparna et al., 2012). 9,12-Octadecadienoic acid (Z,Z) (Linoleic acid), one of the polyunsaturated fatty acid, has been reported able to prevent DM and attenuate oxidative stress in diabetic condition (Suresh and Das, 2003). Linoleic acid has been shown able to prevent experimentally-induced DN as a result of exposure to high glucose levels (Pitel et al., 2007).

Fig. 7. (A): Immunoperoxidase images showing distribution of Ki-67 (B) Quantification of Ki-67 expression. Data were represented as number of positively stained cells/image. (C) Western blot showing PCNA expression. (D) Pcn mRNA levels. *p < 0.05 compared to NC, †p < 0.05 compared to D. Data were expressed as mean ± S.E.M from 6 animals per treatment. Scale bar represents 50 µm.
Besides, linoleic acid (Z,Z) has been found to possess anti-inflammatory activities (Jones, 2002).

In conclusions, the present study has provided scientific cues to support the use of PN in ameliorating progression of nephropathy in DM. By having these effects, PN is useful to decrease DN progression to chronic renal failure, effects which might be contributed by its bioactive compounds. However, effects of individual compounds need to be further explored. These findings therefore justify the claims that this herb is useful in treating genito-urinary disorders and pave the way for its use as an agent to treat DN.

Fig. 8. (A): Chromatogram showing peaks with retention time (x-axis [Retention time; y-axis [% intensity/% abundance). (B) GCMS analysis of the major compounds in PN.
Table 3
The active principles of PN and their retention time (RT), molecular formula and molecular weight (MW).

<table>
<thead>
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<th>No.</th>
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<th>Molecular formula</th>
<th>Molecular Weight</th>
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<td>C9H7NO</td>
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<tr>
<td>5</td>
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<td>9,12-Octadecadienoic acid (ZZ)</td>
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</tr>
<tr>
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<tr>
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Conflicts of interest statement

Authors declare no existing conflict of interest.

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References


