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Original Article

Myrciaria cauliflora extracts attenuate diabetic nephropathy involving the Ras signaling pathway in streptozotocin/nicotinamide mice on a high fat diet

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ABSTRACT

Diabetic nephropathy (DN) is a major cause of end-stage renal disease and its mortality is continuously increasing worldwide. Previous studies indicate that reactive oxygen species play an important role in high glucose-induced renal injury. *Myrciaria cauliflora* has been reported as a functional food rich in anthocyanins possessing anti-oxidative and anti-inflammatory properties. This study examined whether *M. cauliflora* extracts (MCE) can attenuate diabetic nephropathy progression in type 2 diabetes mellitus mice. First, the composition of the anthocyanins and polyphenols of MCE were determined by high-performance liquid chromatography and spectrophotometry. One hundred mg/kg of streptozotocin and 240 mg/kg nicotinamide were administered to C57BL/6J mice fed a high fat diet and varied concentrations of MCE. The plasma glucose concentration, body weight, oral glucose tolerance, blood pressure, renal ultrasound ultrasonic wave were monitored every 2 weeks. Following euthanasia, the kidneys of the mice were analyzed using hematoxylin–eosin, periodic acid Schiff, Masson's trichrome, and immunohistochemistry staining. The results showed that MCE stabilized the plasma glucose and indirectly improved insulin sensitivity in diabetic mice. In addition, diabetes-caused glomerular atrophy, accumulation of saccharide, and formation of collagen IV were recovered or reduced under treatment with MCE in diabetic mice. Our results indicate that MCE has beneficial effects in DN and the mechanism has been confirmed to inhibit Ras/PI3K/Akt and kidney fibrosis related proteins. This work illustrates the potential of MCE rich in anthocyanins and polyphenols as a natural food to inhibit DN.

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1. Introduction

Diabetic nephropathy (DN) is a pathological progression from hyperfiltration to microalbuminurea, then to macroalbuminurea, and finally to renal failure [1]. A typical morphological change in the diabetic kidney involves the accumulation of extracellular matrix (ECM) in glomeruli that correlates with the loss of renal function such as mesangial expansion, tubulointerstital fibrosis, and irreversible deterioration [2,3]. Under diabetic conditions, the synthesis of matrix proteins including collagen IV and fibronectin are upregulated in mesangial cells, ultimately leading to glomerulosclerosis [3,4]. The boosted production of matrix proteins can be responsible for hyperglycemia-associated accumulation of ECM constituents at the glomerular levels [5,6].

In addition to high glucose, Ras/Rho are stimulated by other components of the diabetic milieu, such as advanced glycation end products (AGEs), reactive oxygen species, the hexosamine pathway and oxidized low-density lipoprotein in renal cells [7–10]. Ras family members are downstream convergence points for signal cascades mediated by many cell surface receptors with ligands expressed in renal injury, such as transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), angiotensin II, and epidermal growth factor (EGF), allowing transduction of the signal to downstream profibrotic effectors. As such, they may be an important potential target for treating the fibrosis of chronic kidney disease [11–14]. As demonstrated by initial studies, the pathway contributes to progrowth, profibrotic/prosclerotic signalling, and enhanced ECM production [7,8]. The effects of high glucose on mesangial cells were inhibited by simvastatin [15], Y27632 (ROCK inhibitor), or fasudil, as well as the transfection of mesangial cells with inactive RhoA mutant or siRNA targeting RhoA. However, nephropathy induced by diabetes involved in Ras signaling is still unclear.

Polyphenols are a large family of antioxidant phytochemicals in our diet. According to the recent literature, the mechanism by which polyphenols express their beneficial properties on DN appears to be through inhibition of glomerular hypertrophy [16]. Studies show that some of those exhibit high biologic potential, since they have higher fiber and antioxidant contents, such as in several phenolic compounds and anthocyanins [17, 18]. Jaboticaba (*Myrciaria cauliflora*), a native fruit from the Brazilian Atlantic Forest, has a peel with interesting nutritional properties. It is a good source of minerals, fibers, and phenolics, especially anthocyanins [19–21]. Jaboticaba is cited in popular medicine as an astringent, useful against diarrhea, skin irritations, gut inflammation, and hemoptysis [22]. Besides, Leite-Legatti et al showed that freeze-dried jaboticaba peel powder increased high-density lipoprotein cholesterol and improved insulin resistance in rats in a diet-induced obesity model [21]. Dragano et al also showed improvements in insulin sensitivity in high–fat-fed mice after intake of the same freeze-dried jaboticaba peel powder [23]. However, these recent studies did not evaluate the preventive effects of jaboticaba peel intake regarding the risk factors for DN. The aim of this study was to determine the effects of *M. cauliflora* water extract (MCE) intake in streptozotocin (STZ)/nicotinamide (NA) mice fed a high-fat diet.

2. Materials and methods

2.1. Preparation and analysis of the compositions of MCE

MCE were prepared from the fruit of *M. cauliflora* Berg, which were obtained from the Modern Garden Jabuticaba Co., Ltd, Changhua, Taiwan. Briefly, the lyophilized fruit (100 g) were macerated and stirred with water (100 mL), and the juice was then filtered and centrifuged (10,000 × *g*, 15 minutes). The extract was filtered and concentrated under reduced pressure at 30°C. Then, the filtrate was lyophilized (−80°C, 12 hours) to obtain MCE and stored at −20°C before use. The concentration of total polyphenol was analyzed according to a Folin–Ciocalteu assay. The MCE (0.1 mg) were dissolved in an eppendorf with 1 mL of distilled water, and Folin–Ciocalteu reagent (2N, 0.5 mL) was then added and mixed for 3 minutes. The mixture had 3 mL of 2% Na_2CO_3 added and it then stood for 15 minutes with intermittent mixing. The absorbance of the mixture was measured at 750 nm using a Hitachi U-3210 spectrophotometer (Hitachi, Tokyo, Japan) with gallic acid as the standard.

2.2. High-performance liquid chromatography and polyphenols content analysis of MCE

The polyphenols were identified by high-performance liquid chromatography (HPLC) analysis using a Hewlett-Packard Vectra 436/33N system with a diode array detector. The 25 mg/mL of MCE were filtered through a 0.22-μm filter disk and injected into the column employing a 5-μm RP-18 column (4.6 mm × 150 mm inner diameter). The eluted polyphenols were monitored at 280 nm, and UV spectra were collected to confirm peak purity. Two solvent systems were used in the mobile phase: A, 2% acetic acid/water; B, 0.5% acetic acid in water/acetonitrile.

2.3. Animal model

Male C57BL/6J mice, age 5–6 weeks, were used in the *in vivo* studies; all mice were handled according to the guidelines of IACUC (Institutional Animal Care and Use Committee), CSMU (Animal Center of Chung Shan Medical University) and kept on a 12 hour light/dark cycle in a temperature-controlled vivarium. They were allowed to take food and water *ad libitum* before any food-related treatment began. Mice were fasted for 16 hours before receiving the chemicals. STZ was dissolved in a 50mM citric acid buffer at the time of use and was administered at 100 mg/kg, intraperitoneally twice on Days 0 and 2. NA was dissolved in saline and injected at 240 mg/kg intraperitoneally at 15 minutes before administration of STZ. The blood sugar (BS) level and body weight were measured every 2 weeks. Biochemical measurements and pathological examinations were performed 8 weeks later.

2.4. Oral glucose tolerance test

Mice were fasted for 16 hours before oral administration of glucose solution (2.0 g/kg), and the BS level was measured at 0 minutes, 15 minutes, 45 minutes, 95 minutes, and 135 minutes following glucose administration at Weeks 0, 2, 4, 6, and 8 after induction of diabetes.

2.5. Measurement of biochemical parameters in blood and urine

Blood was collected from the tail vein of the mice every 2 weeks after beginning the induction of diabetes. BS concentration was measured 16 hours after removal of food at 7 PM as a 'fasting' BS level, using a blood glucose meter (Accu-Chek Performa; Roche, Nutley, NJ, USA). Immediately after measuring the BS concentration, food was given to mice. For urine collection, mice were transferred to clean metabolic cages (one mouse per cage). Total protein, creatinine and albumin in the urine were determined by a Mouse Albumin ELISA Kit (Bethyl Laboratories Inc., Montgomery, TX, USA) and QuantiChromCreatinine Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's procedures. Mouse urinary albumin/urinary creatinine ratio was calculated as μg/mg [24].

2.6. Renal morphology assessment and immunohistochemistry analysis

Hematoxylin–eosin, periodic acid–Schiff (PAS), and Masson's trichrome stainings were performed by conventional histochemical methods described previously [25]. After the mice were euthanized, full-thickness biopsies were taken and fixed in 4% formaldehyde, and then embedded in paraffin. Tissue slices were subjected to hematoxylin–eosin staining. For PAS staining, the kidneys were harvested and fixed in 10% formalin; 5-μm thick sections were stained with PAS reagent. Immunohistochemistry was performed in paraffin sections and detected in cortical sections with a specific primary antibody and the ABC staining kit, and visualized with the DAB detection kit (both kits from Vector Laboratories Inc., Burlingame, CA, USA). The primary antibody used in the present study was RAS (Sigma; Maixin, Fuzhou, China). After being incubated with the secondary antibody (Proteintech Group, Chicago, IL, USA), 2-μm thick sections were developed with an SP immunohistochemical kit (Maixin, China) to produce a brown product and counterstained with hematoxylin. Histologic evaluation was performed using a Nikon Eclipse E600 microscopy system (Nikon Instruments Inc., Melville, NY, USA) without knowledge of the identity of the various groups. The slides were coded and examined by a pathologist blinded to the study protocol to identify histological alterations.

2.7. Measurement of cell proliferation

A murine kidney mesangial cell line (MES13) was obtained from BCRC (Bioresource Collection and Research Center, Hsinchu, Taiwan) and grown in a 3:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium containing 5% (v/v) fetal bovine serum, 4mM L-glutamine, 1mM penicillin/streptomycin, and 5.5mM glucose. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2–95% air. The cell proliferation assay was performed using the MTT [3-(4, 5-dimethylthiazol-2-vl)-2, 5-diphenyl tetrazolium bromide] assay. MES13 cells were seeded and treated with MCE of 0.2 mg/mL, 0.4 mg/mL, and 0.6 mg/mL for 1 day, 2 days, or 3 days in 25mM high glucose medium conditions. The mediums from all treatments were removed, and the cells were washed with phosphate-buffered saline. After adding fresh medium, the cells were incubated with 20 mL MTT (5 mg/mL) for 4 hours. Then, the blue crystals, which are the metabolized products of MTT, were extracted by isopropanol. Absorbance at 563 nm was measured and used for the proportion of proliferating cells.

2.8. Western blot analysis

Immunoblot analysis was performed according to standard protocols. After resolution on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, proteins were electroblotted onto an immobiolon-NC (nitrocellulose) membrane that was subsequently blocked with 1% (w/v) nonfat milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST). The membranes were washed with TBST and incubated with the indicated primary antibody in TBST. After extensive washing with TBST, the blot was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Band detection was revealed by enhanced chemiluminescence (ECL) using ECL western blotting detection reagents and exposure to ECL hyperfilm on a FUJIFILM Las-3000 system (FUJFILM Co., Tokyo, Japan). The proteins were then quantitatively determined by densitometry using FUJFILM-Multi Gauge V2.2 software.

2.9. Statistical analysis

Statistical analysis was performed with Sigmaplot software (version 12), and descriptive statistics are presented as mean ± standard deviation. The difference among the three groups (control, STZ/NA-induced, and MCE groups) was analyzed by one-way ANOVA. The difference between two groups was analyzed by Student *t* test.

3. Results

3.1. Polyphenol content in MCE

After water extraction, the yield of MCE was approximately 2.5% and the phytochemical profile was analyzed by HPLC and spectrophotometry (Figure 1). According to the calibration standards, the major polyphenol of MCE was protocatechuic acid (73.82%), and the other polyphenolic constituents of MCE were gallic acid (9.57%), gallocatechin (9.32%), catechin (4.00%), and rutin (3.28%) as analyzed by

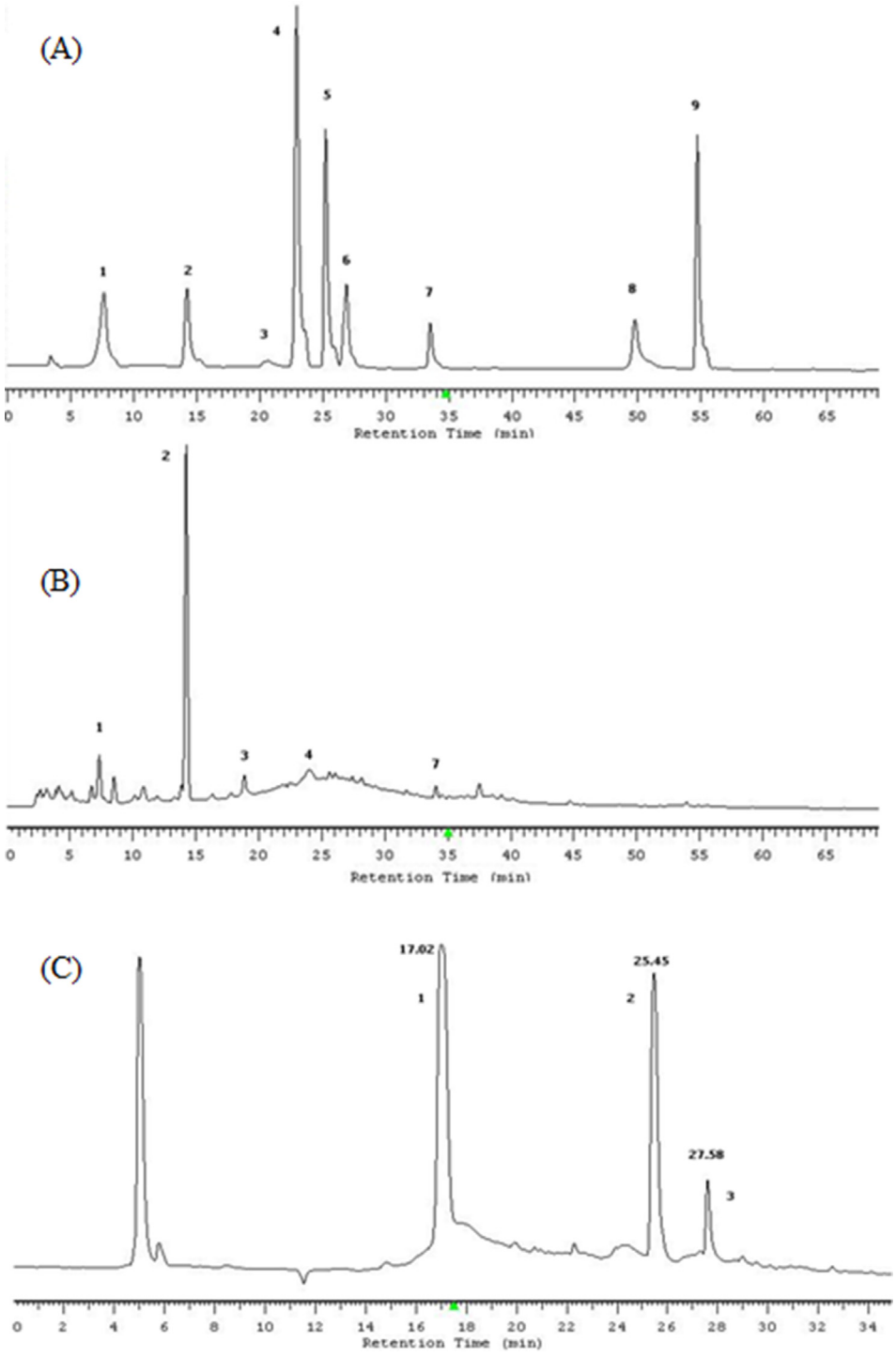

Fig. 1 – High-performance liquid chromatogram of *Myrciaria cauliflora* extract (MCE). (A) Polyphenol contents of nine polyphenols standards (1mg/mL;10μL) are: 1. gallic acid, 2. protocatechuic acid, 3. catechin, 4. gallocatechin, 5. gallocatechin gallate, 6. caffeic acid, 7. rutin, 8. quercetin, 9. naringenin. (B) Polyphenol contents of MCE (10 mg/mL; 10μL). (C) Anthocyanin contents of MCE. Retention time for: delphinidin, 16.54 minutes; cyanidin, 25.75 minutes; peonidin, 27.56 minutes.

HPLC (Table 1). The HPLC chromatogram shows the presence of several unidentified peaks. The main anthocyanins of MCE were delphinidin (58.08%), cyanidin (14.94%), peonidin (10.70%), and unknown components with our standards (Table 2).

3.2. *MCE mitigated BS and renal injury in diabetic mice*

The BS of the mice was monitored by OGTT every 2 weeks. MCE could stabilize the fasting BS of diabetic mice induced by STZ/NA for the whole experiment (Figure 2A). In addition,

Table 1 – Identification of polyphenol content in *Myrciaria cauliflora* extract.

Polyphenol	%	mg/100 g of FW	
		Dry	Fresh
Gallic acid	9.57%	96	2.4
Protocatechuic acid	73.82%	738	18.45
Catechin	4.00%	400.1	10.0025
Gallocatechin	9.32%	93	2.325
Rutin	3.28%	33	0.825

FW = weight of *M. cauliflora* fruit.

high BS was detected in STZ/NA mice at 2 weeks and 1% MCE significantly ameliorated the high BS level after treatment for 6 weeks (Figure 2B), indicating the effect of MCE in regulating BS. The STZ/NA mice caused slight weight gain, significant hyperlipidemia, and high blood urea nitrogen (BUN), creatinine, and blood pressure, as shown in Table 3. MCE improved STZ/NA-induced hyperlipidemia-induced by decreasing triacylglycerol and total cholesterol levels. MCE also had an inhibitory effect on the BUN, blood creatinine, and blood pressure. However, there was no difference between the STZ/NA-induced and MCE treatment groups as to the urine creatinine level (data not shown). Then the urine albumin level was determined. The ratio of urine albumin/urine creatinine, an indicator of renal dysfunction, increased in the STZ/NA group and could be alleviated by MCE treatment.

3.3. *MCE decreased the ECM accumulation of glomeruli in STZ/NA mice*

ECM accumulation is one of the characteristic pathological alterations in the glomeruli of diabetic nephropathy patients. Both collagen and fibronectin are the predominant components of ECM and generally considered the predictors of ECM accumulation. Representative glomerular atrophy and mesangial matrix expansion were observed in the STZ/NA group compared with the control group, and MCE ameliorated glomerular atrophy and mesangial matrix expansion induced by STZ/NA (Figure 3A). Figure 3B shows the quantification of mesangial matrix expansion. The mesangial matrix index, calculated by the white circle area in the tuft area, increased in the STZ/NA group and was significantly reduced by MCE exposure.

STZ/NA also induced significant kidney damage, as revealed by PAS staining. PAS staining is most useful for analyzing the glomerulus, since it delineates the glomerular cells, mesangial matrix and potential expansion in great detail, as well as potential modifications of the composition of the matrix, changes in the glomerular basal membrane, i.e.

Table 2 – Identification of anthocyanins from jaboticaba.

Anthocyanin	%	mg /100 g of weight	
		Dry	Fresh
Delphinidin	58.08%	581	14.5
Cyanidin	14.94%	149	3.725
Peonidin	10.70%	107	2.675

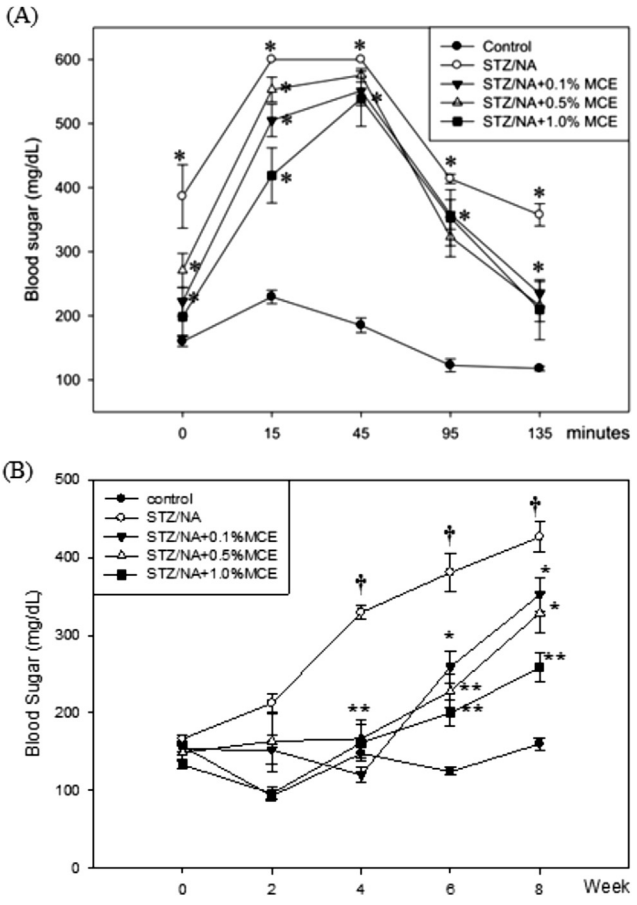

Fig. 2 – Fasting blood sugar level in the mice from Week 0 to Week 8. After treatment with streptozotocin/nicotinamide (STZ/NA), mice were fed with high fat diet and three concentrations of *Myrciaria cauliflora* extract (MCE), this fasting blood glucose of mice were tested every 2 weeks. Data are shown as mean ± standard deviation; $n = 10$ mice per group. †$p < 0.05$ versus control group; *$p < 0.05$, **$p < 0.001$ versus STZ/NA group.

thickening, irregularities, doubling, rupture, and finally fibrinoid necrosis of the glomerular tuft. Histological examination and quantification revealed prominent nodular glomerulosclerosis with glomerular basement membrane thickening indicated by the arrow. This injury was attenuated dose-dependently by MCE treatment in mice, proving MCE has the potential to ameliorate diabetic nephropathy (Figure 4).

Changes in mesangial and tubulointersticial fibrosis indicated by Masson's trichrome staining are shown in Figure 5. Increased collagen deposition observed in the STZ/NA group compared with control group was inhibited by MCE in a dose dependent manner.

3.4. *MCE decreased Ras-related protein expression in STZ/NA mice*

The Ras pathway is activated in renal cells exposed to diabetic milleu, indicating a candidate role of Ras in DN [9,15]. Using

Table 3 – Biochemical characteristics after 8 weeks of treatment with *Myrciaria cauliflora* extract (MCE) in streptozotocin/nicotinamide (STZ/NA) mice.

	Control	STZ/NA	STZ/NA + 0.1% MCE	STZ/NA + 0.5% MCE	STZ/NA + 1.0% MCE
BW (g)	22.5 ± 1.50	24.3 ± 0.60	24.7 ± 0.80	25.5 ± 0.96	24.9 ± 1.00
BUN (mg/dL)	17.43 ± 0.77	29.21 ± 1.01†	26.37 ± 0.91*	25.81 ± 1.31*	24.11 ± 0.57*
Cre (mg/dL)	0.21 ± 0.03	0.33 ± 0.02†	0.25 ± 0.02*	0.2 ± 0.03*	0.18 ± 0.02**
HDL/LDL	7.05 ± 0.57	7.52 ± 0.70	4.32 ± 0.51*	8.43 ± 0.99	5.81 ± 0.69**
TG (mg/dL)	55.86 ± 8.10	71.71 ± 4.89†	63.67 ± 2.17*	65 ± 5.72*	64.88 ± 4.85*
TC (mg/dL)	82.22 ± 4.17	119.29 ± 6.13†	107.78 ± 4.15	97.83 ± 5.61*	79.22 ± 4.43*
BP (mmHg)	90 ± 5.03	101 ± 1.15†	91.83 ± 2.47*	94.8 ± 2.96*	76.5 ± 3.62*
UACR (μg/mg)	33.2 ± 2.5	100.3 ± 6.3†	89.1 ± 5.7*	65.6 ± 2.7*	44.9 ± 2.1**

Data are presented as mean ± SD; $n = 10$ mice per group. †$p < 0.05$, significant difference compared to the control group determined by Student's t test.*$p < 0.05$, and **$p < 0.001$, significant difference compared to the STZ/NA group determined by ANOVA.
BP = blood pressure; BUN = blood urine nitrogen; BW = body weight; Cre = creatinine; HDL = high-density lipoprotein; LDL = low-density lipoprotein; TC = total cholesterol; TG = triacylglycerol; UACR = urinary albumin/urinary creatinine.

immunohistochemistry staining in this study, higher expression of Ras protein was observed in the STZ/NA treated group, which was as expected. The activated Ras in the mice samples was decreased in the MCE treatment groups (Figure 6). As previous research showed, Ras activation was associated with Akt activation [26]. Further details of the mechanism are analyzed as follows.

3.5. *Effect of MCE on high glucose-induced mesangial cell proliferation*

The cytotoxicity effect of MCE on MES13 mesangial cells was determined first. Cell viability of 100–85% with MCE at 0–1 mg/mL revealed no cytotoxicity to MES13 cells (data not shown). MCE concentrations of 0.2 mg/mL, 0.4 mg/mL, and 0.6 mg/mL were selected for the following experiment. In the MTT assay, stimulation with high glucose (25mM) increased MES13 cell proliferation compared with the control (5.5mM glucose) group. MCE (0.2 mg/mL, 0.4 mg/mL, and 0.6 mg/mL) inhibited high glucose-induced cell proliferation dose dependently (Figure 7).

3.6. *Interruption of high glucose-induced mesangial fibrosis signaling by MCE*

The levels of collagen, fibronectin, and Ras related pathway proteins assayed in the animal model were determined *in vitro*. In Figure 8, when MES13 cells were treated with 25mM

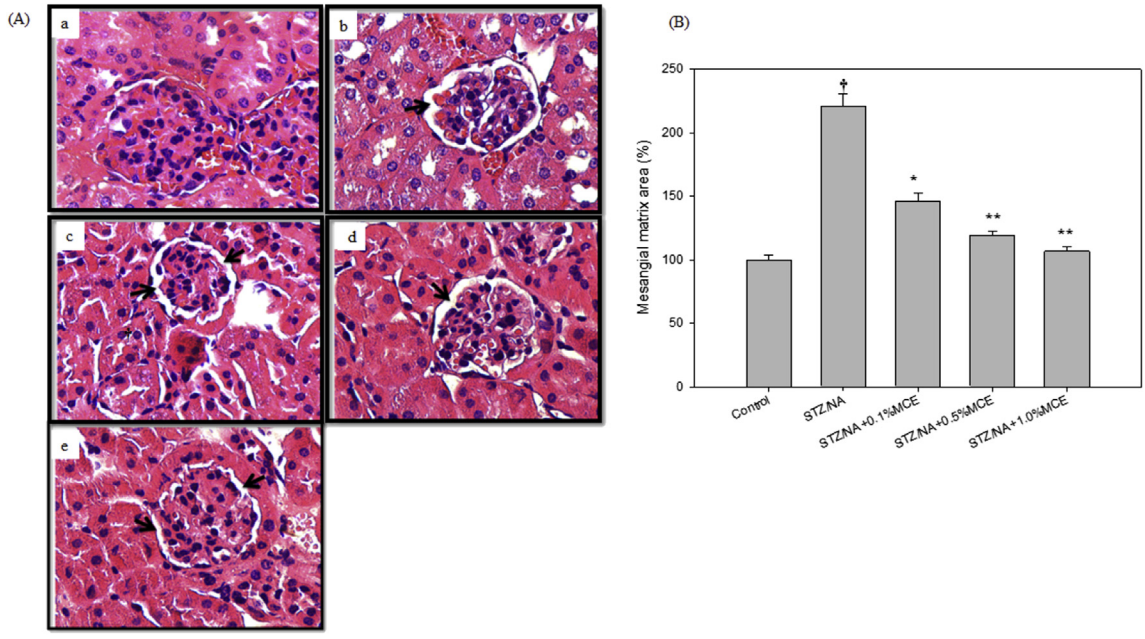

Fig. 3 – *Myrciaria cauliflora* extract (MCE) decreases mesangial matrix expansion in the glomerulus. (A) Kidney slices were embedded in paraffin, stained with hematoxylin and eosin. Light photomicrographs (400× magnification) of kidney sections from control group (a), streptozotocin/nicotinamide (STZ/NA) group (b), STZ/NA + 0.1% MCE group (c), STZ/NA + 0.5% MCE group (d), and STZ/NA + 1.0% MCE group (e). Arrow indicates white circle, the location of mesangial matrix expansion in the glomerulus. (B) Quantification of the mesangial matrix area. Three randomly selected glomeruli per mouse were examined. Data are shown as mean ± standard deviation; $n = 10$ mice per group. †$p < 0.05$ versus control group; *$p < 0.05$, **$p < 0.001$ versus STZ/NA group.

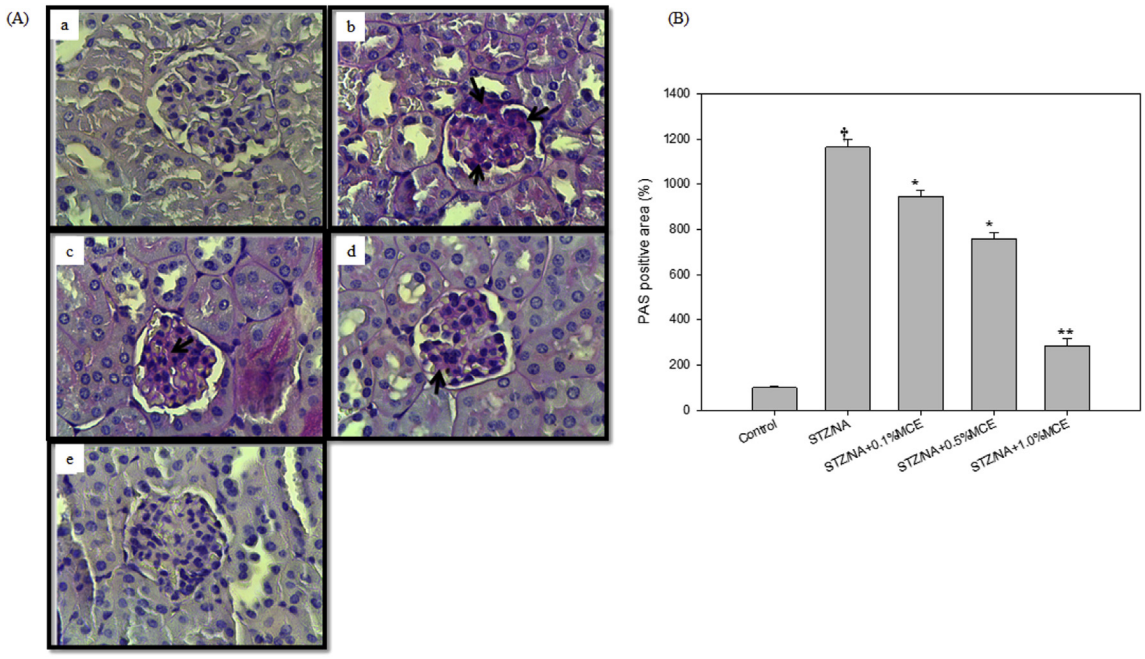

Fig. 4 – *Myrciaria cauliflora* extract (MCE) decreases accumulation of saccharide in the glomerular periphery. Periodic acid–Schiff staining was used to detect accumulation of saccharide. (A) Light photomicrographs of kidney sections from the control group (a), streptozotocin/nicotinamide (STZ/NA) group (b), STZ/NA + 0.1% MCE group (c), STZ/NA + 0.5% MCE group (d), STZ/NA + 1.0% MCE group (e) were displayed (400× magnification). Arrow indicates mauve color, the location of saccharide accumulated in the glomerulus. (B) The accumulation of saccharide index, calculated by the periodic acid–Schiff-positive area in the tuft area, was significantly increased in STZ/NA group as compared with the other four groups. Three randomly selected glomeruli per mouse were examined. Data are shown as mean ± standard deviation; $n = 10$ mice per group. †$p < 0.05$ versus control group; *$p < 0.05$, **$p < 0.001$ versus STZ/NA group.

glucose, collagen was slight and fibronectin was significantly increased, whereas MCE attenuated both proteins levels under high glucose conditions. In addition, as expected, MCE inhibited Ras and p-Akt levels induced by high glucose. A previous study demonstrated that phosphoinositide 3-kinase (PI3K) activation was a crucial event for the induction of ECM protein in mesangial cells [13], and we also investigated the influence of high glucose on PI3K activity. High ambient glucose induced a significant increase in p-phosphoinositide 3-kinase (p-PI3K) activity and MCE dose dependently decreased p-PI3K expression. Taken together, our findings demonstrate that MCE ameliorates mesangial fibrosis, at least in part, via the Ras/PI3K/Akt signaling pathway.

4. Discussion

DN is one of the major complications of diabetes mellitus and a leading cause of end-stage renal failure in the world today. Ras is activated and regulates both cell proliferation and TGF-β-induced epithelial-mesenchymal transition during renal fibrosis. In this study, MCE ameliorating the structural and functional abnormalities of the diabetic kidney in mice might be associated with inhibition of Ras regulated renal fibrosis.

The STZ-induced diabetic animal model has been widely used in DN research. STZ is a chemical inducer used for type 1 diabetes through producing free radicals in the body, specifically cutting DNA chains in the pancreatic beta cells, and resulting in disorder in the function and destruction of the β cells [27]. Nakamura et al established that the STZ/NA diabetic model possesses characteristics quite similar to type 2 nonobese diabetes, which constitutes a majority of East Asian diabetic patients [28]. By supplement with NAD, the progress of insulin-dependent diabetes could be prevented [29]. When given the calorie-controlled high fat diet, the STZ/NA mice caused hyperlipidemia, and significantly increased insulin resistance. The diabetic mice model was used in the present study and the STZ/NA group exhibited significant hyperlipidemia, as well as high BUN, creatinine, blood pressure, and fasting blood sugar compared with the control group (Table 3, Figure 2). In addition, renal dysfunction including ECM accumulation, mesangial cell matrix deposition, and glomerular basement membrane thickening were found in the kidney sections of the STZ/NA group. Interestingly, MCE attenuated the morphological alterations and improved renal function, indicating MCE could reduce the risk of renal dysfunction by preventing injury to the structure and cellular basis.

Glomerular mesangial cells and interstitial fibroblasts are renal cells with an essential role in the development of renal fibrosis [30,31]. Proliferation of renal fibroblasts has been implicated in the pathophysiology of interstitial fibrosis, since fibroblast activation leads to the myofibroblast phenotype and a subsequent increase in extracellular matrix deposition. Hyperglycemia and TGF-β are the pathogenic factors for

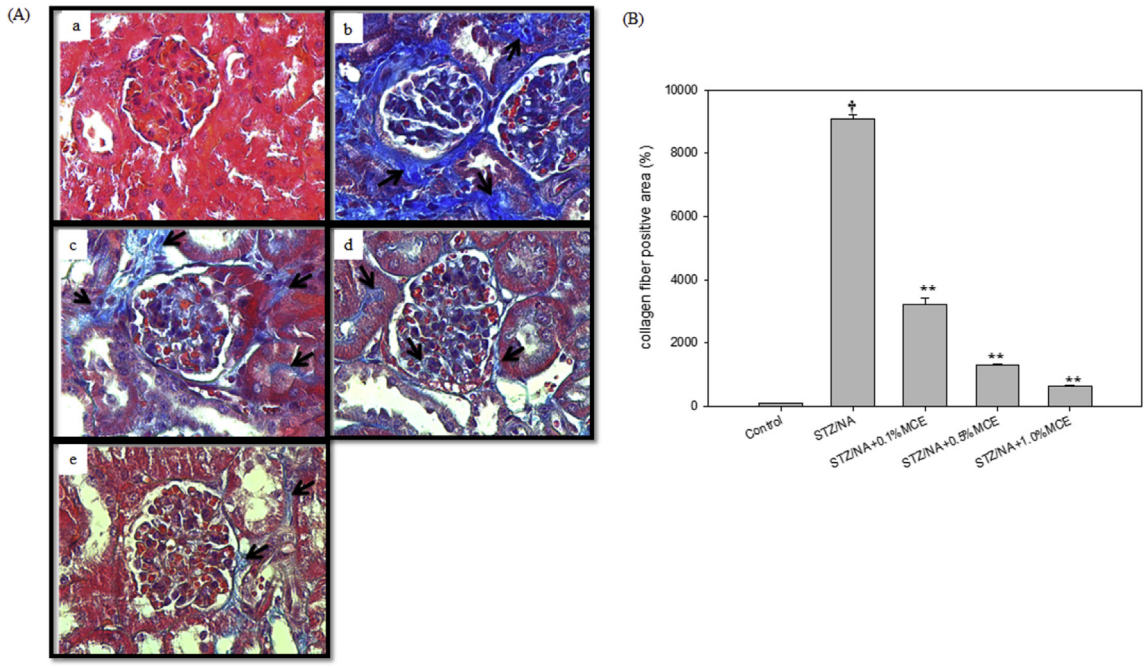

Fig. 5 – *Myrciaria cauliflora* extract (MCE) reduces collagen fiber expansion in the glomerular periphery. (A) Light photomicrographs of kidney sections from control group (a), streptozotocin/nicotinamide (STZ/NA) group (b), STZ/NA + 0.1% MCE group (c), STZ/NA + 0.5% MCE group (d), STZ/NA + 1.0% MCE group (e) stained by Masson's trichrome reagent were displayed (400×magnification). Arrow indicates blue color, the location of collagen fiber expansion in the glomerulus. (B) The collagen fiber expansion, a kidney injury marker, was calculated by the Masson's trichrome-positive area in the tuft area. Data are shown as mean ± standard deviation; $n = 10$ mice per group. †$p < 0.05$ versus control group; *$p < 0.05$, **$p < 0.001$ versus STZ/NA group.

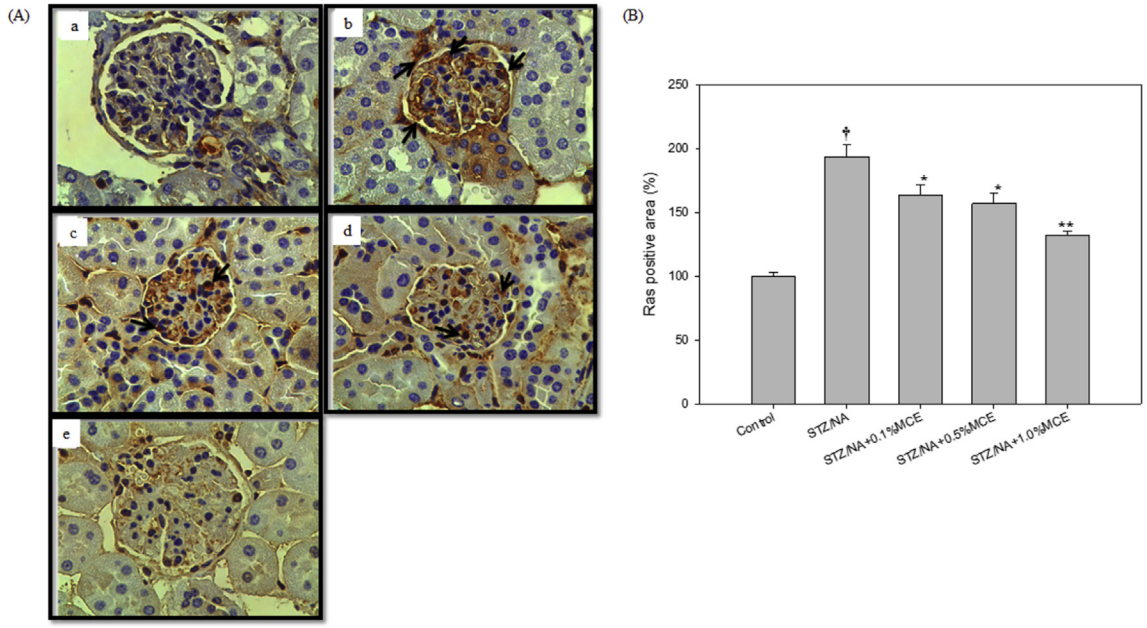

Fig. 6 – *Myrciaria cauliflora* extract (MCE) decrease the Ras expression. (A) Light photomicrographs of kidney sections from control group (a), streptozotocin/nicotinamide (STZ/NA) group (b), STZ/NA + 0.1% MCE group (c), STZ/NA + 0.5% MCE group (d), STZ/NA + 1.0% MCE group (e) were displayed Ras level using IHC staining (400× magnification). Arrow indicates the location of Ras expression. (B) The Ras protein expression significantly increased in STZ/NA group as compared with the other group. Data are shown as mean ± standard deviation; $n = 10$ mice per group. †$p < 0.05$ versus control group; *$p < 0.05$, **$p < 0.001$ versus STZ/NA group.

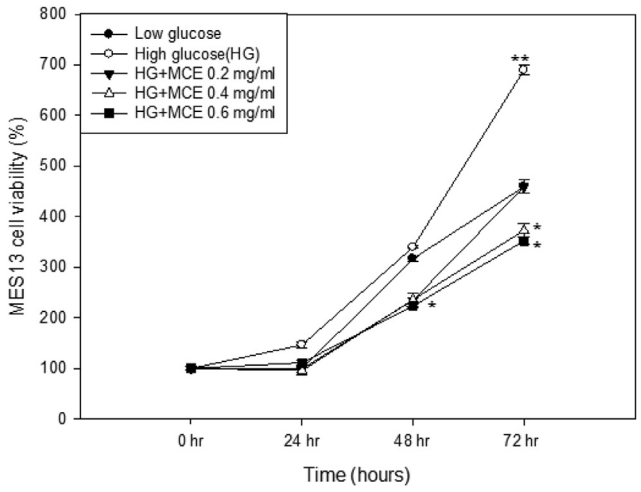

Fig. 7 – The effect of *Myrciaria cauliflora* extract (MCE) on MES-13 cells proliferation induced by high glucose. MES-13 cells were induced by 25mM glucose medium and treated with indicated concentration of MCE for 24 hours, 48 hours, and 72 hours. The 5.5mM glucose medium cultured cells were as control group. Cell viability was measured by 3-(4, 5-dimethylthiazol-2-vl)-2, 5-diphenyl tetrazolium bromide assay. The data the means ± standard deviation from three replicates per treatment. *$p < 0.05$, **$p < 0.001$, significant difference compared to the low glucose (control) group determined by Student t test.

diabetic nephropathy. Besides, many of the cytokines, growth factors, and integrins involved in proliferation are known to activate intracellular signaling pathways that converge on Ras proteins [32]. Our results showed high level expression of Ras protein in both STZ/NA-induced group mice and MES13 cells treated with high glucose, whereas MCE treatment attenuated this increase, suggesting that MCE improved renal injury by regulating the Ras pathway to reduce extracellular matrix deposition such as collagen and fibronectin.

There is a close relationship between TGF-β and Ras signaling: TGF-β overcomes the Ras mitogenic effects, and Ras can counteract TGF-β signaling, altering the expression of the TGF-β receptor. By contrast, renal Ras activation serves as an intracellular signal transduction pathway for molecules involved in obstructing kidney damage, such as TGF-β [33]. Interestingly, data from our other study revealed that MCE could offer a prophylactic role against diabetic nephropathy through inhibiting oxidative stress and inflammatory response (unpublished data).

Thousands of studies have been conducted on better treating and improving DN. Unfortunately, the pathogenesis of DN remains unclear, and effective and safe therapies are in great demand. Rather than conventional medicines that concentrate on delaying renal failure by controlling BS, blood pressure, and/or ameliorating microalbuminuria, BUN, and creatinine, plant-based food rich in phytochemical or edible traditional Chinese medicine emphasize the patients' overall inner system and focus on repairing the kidney with consideration of overall health. Recently, the anti-DN activity of lignans from edible plants has been investigated. For example, cinnamaldehyde from *Cinnamomum zeylanicum* has been shown to exert protective effects against alloxan-induced

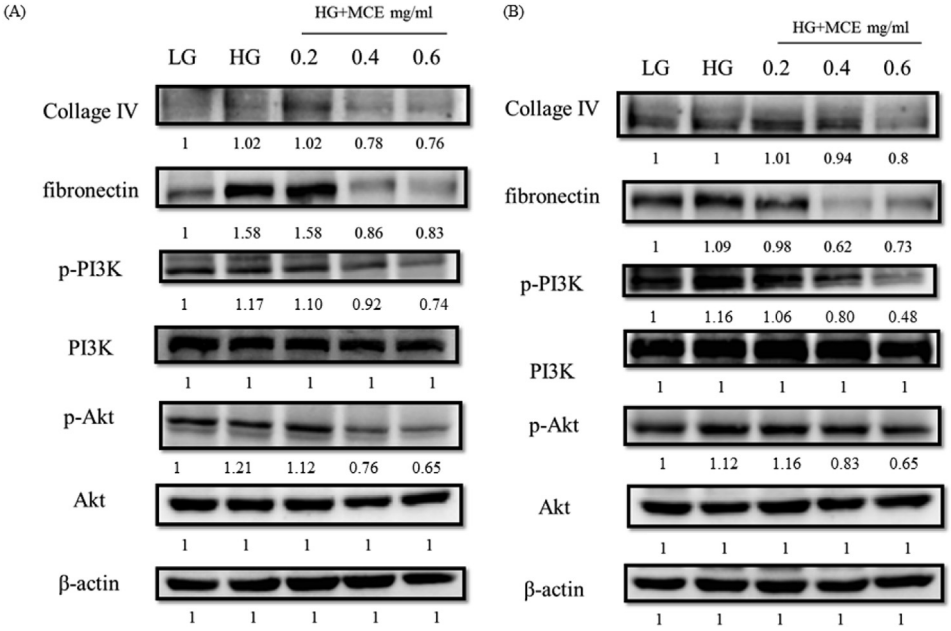

Fig. 8 – *Myrciaria cauliflora* extract (MCE) inhibited the proliferation and fibrosis proteins expression of MES-13 cells induced by high glucose. High glucose medium (25mM) was used to induce the proliferation in MES-13 cell, which were treated with the indicated concentration of MCE for (A) 48 hours and (B) 72 hours, and cells treated with low glucose medium (5.5mM) were as a control. Protein expression was detected by western blot analysis and quantified by densitometry. The data were representative of three independent experiments with comparable observations.

renal damage by reducing glomerular expansion [34]. Flavonoids with anti-DN effects are commonly found in edible plants such as green tea (*Camellia sinensis*), rooibos tea (*Aspalathus linearis*), and lingonberry (*Vaccinium vitisidaea*) [35–37]. Recent research using STZ induced rats as a diabetic model revealed renal function, blood glucose and glycated protein profiles were improved in rats supplemented with green tea in the drinking water. There are reports glomerular hypertrophy being inhibited through consumption of herbal antioxidants extracts such as garlic, ginger [38], and *Ginkgo biloba* [39] in diabetic animals. In addition, glomerular hypertrophy has been inhibited by antioxidants such as vitamin E and α-tochopherol in diabetic rats [40,41]. Anthocyanins are widely distributed in human diets, suggesting that we ingest large amounts of anthocyanins from plant-based foods. Anthocyanins are a class of flavonoids with high antioxidant ability, responsible for the bright colors visible in most fruits and vegetables. Many researchers have focused on the protective effects of grape proanthocyanidins against DN. It was discovered the proanthocyanidins could reduce serum AGEs, proteinuria, and systolic blood pressure, decrease the receptor of advanced glycation end-products and connective tissue growth factor expression as well as oxidative stress in the kidney, while increasing renal antioxidant enzyme activity [42,43]. Purple corn has been classified as a functional food rich in anthocyanins, dampening high-glucose-induced mesangial fibrosis and inflammation [44]. *M. cauliflora* has been reported to be rich in phenolic constituents, including resorcinol, p-hydroxybenzoic acid, anthocyanins, hydroxycinnamic acids, flavonoids, coumarins, and ellagitannins. Phenolic compounds are well-known to be potent deactivators of reactive species, having strong antioxidant and anti-inflammatory biological properties [45], as well as anticancer potency [46,47]. This is the first study showing the potential of MCE as a natural food to inhibit DN.

Conflicts of interest

The authors declare that they have no competing interests.

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