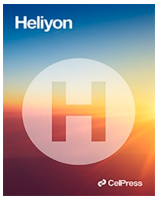
Heliyon 10 (2024) e33217

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Bioflavonoid combination attenuates diabetes-induced nephropathy in rats via modulation of MMP-9/TIMP-1, TGF-β, and GLUT-4-associated pathways

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ARTICLE INFO

Keywords:
Diabetic nephropathy
Natural products
Medicinal plants
MMP-9
TIMP-1
GLUT-4
TGF-β
Bioflavonoids

ABSTRACT

Background: Diabetic nephropathy represents a significant microvascular complication of diabetes, characterized by extracellular matrix accumulation, loss of cell-cell junctions, microalbuminuria, and diminished creatinine clearance. Despite its prevalence, therapeutic options dedicated to this condition are currently lacking. Natural products like bioflavonoids have garnered attention for their potential therapeutic benefits. The present study aimed to evaluate the efficacy of a bioflavonoid combination, including ginger extract, soy extract, and hesperetin, in a diabetic rat model.

Methods: Diabetes was initiated in the rat pups via intraperitoneal injection of streptozotocin on the fifth postnatal day. After six weeks, rats exhibiting blood sugar levels exceeding 160 mg/dL were allocated into diabetic control and treatment groups, with eight animals each. A subset of rats received citrate buffer as a control. The treatment group received the bioflavonoid combination orally for twenty-four weeks. Various parameters, including glycemic levels, urinary parameters, antioxidant status, mRNA expression via Western blot, gel zymography, and immunohistochemistry, were assessed at the study's conclusion.

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https://doi.org/10.1016/j.heliyon.2024.e33217
Received 14 March 2024; Received in revised form 24 May 2024; Accepted 17 June 2024
Available online 22 June 2024

Results: The bioflavonoid combination demonstrated significant reductions in hyperglycemia and various urinary parameters compared to controls. Notably, it modulated MMP-9/TIMP-1 expression, upregulated GLUT-4, and downregulated TGF-β. Additionally, the combination enhanced total antioxidant capacity, indicating potential antioxidative benefits.
Conclusions: This study highlights the therapeutic potential of a bioflavonoid combination (ginger extract, soy extract, and hesperetin) in improving renal function in diabetic nephropathy. By modulating key factors such as MMP-9/TIMP-1, TGF-β, and GLUT-4, this combination presents a promising avenue for further exploration in managing diabetic nephropathy. These findings underscore the importance of natural products as potential therapeutic agents in addressing diabetic complications.

1. Introduction

Diabetic nephropathy (DN), driven by chronic hyperglycemia, is the foremost cause of chronic kidney disease (CKD). As per the estimate, around 285 million people are suffering from diabetes-induced CKD. Surprisingly, this figure accounts for 30–50 % of total cases of CKD. Unfortunately, the incidence is projected to increase by 69 % in high-income countries and by 20 % in low-to middle-income countries [1].

Although the mechanisms underlying the development of DN are complex, broadly recognized theories include the activation of oxidative stress, pro-inflammatory state, and fibrosis [2]. Morphological changes have been seen in all the compartments of the kidney, including the glomerulus, tubulointerstitial, and intrarenal veins. Hypertrophy, glomerulus lesions, and basement membrane thickening occurred in the early stages of DN [3,4].

Among many pathologies, matrix metalloproteinases (MMPs) are zinc-dependent proteases that modulate the extracellular matrix (ECM). Overproduction and proliferation of ECM in the kidney are pathological hallmarks of DN [5]. There is a balance between ECM production and degradation. If there is any imbalance in it, this leads to DN. MMPs play a significant role and are responsible for the degradation of ECM [6]. Tissue inhibitors of metalloproteinases (TIMPs) are the natural inhibitors of MMPs and maintain a delicate balance for the normal functioning of the organs.

It is well established that reactive oxygen species (ROS) formation by hyperglycemia in renal cells leads to fibrosis, among other parameters. There is a defined interplay between the ROS and transforming growth factor-β (TGF-β), affecting each other via feedback mechanisms [7].

One of the sensitive components of the glomerular filtration system is podocytes, as they are non-dividing cells [8]. Hyperglycemia-induced damage to the podocytes is well documented. Majorly, podocytes depend on the facilitative diffusion of glucose via GLUT-1 and GLUT-4, insulin-dependent transporters [9]. Insulin-mediated translocation of glucose transporters via IRS-PKB-Akt is well studied. Moreover, hyperglycemia has been held responsible for the downregulation of glucose transporters [10].

New research on preventing progressive renal failure has concentrated on natural products and dietary polyphenols. Flavonoids, a class of natural chemicals, exhibit exceptional pharmacological properties, including antidiabetic, anti-inflammatory, antioxidant, and antihypertension actions [11,12].

This study aimed to explore the effectiveness of combining bioflavonoids, such as soy extract, ginger extract, and hesperetin, in neonatal rats with induced type 2 diabetes at five days postnatal. Glycemic, urinary, biochemical, and molecular parameters were evaluated at the end of the twenty-four-week study period. Until December 22, 2023, there was no literature on PubMed when we used the search terms “soy extract,” “ginger,” “hesperetin,” and “nephropathy” and explored for literature having these search terms in their title or abstracts.

2. Material and methods

2.1. Bioflavonoids

Hesperetin with ≥98 % assay-proven purity was purchased from Cayman Chemicals (Michigan, USA). Dry powder ginger extract with ≥5 % HPLC-proven gingerol was purchased from Sunpure Extracts Private Limited, India. Dry powder soy extract with ≥40 % HPLC-proven isoflavone was purchased from Vital Herbs, India.

2.2. Animal

Five-day-old pups were used for the study. The pups received a single intraperitoneal injection of streptozotocin (STZ) at 90 mg/kg. A few pups were injected with citrate buffer that later served as standard control. Six weeks post STZ injection, animals showing fasting blood glucose ≥160 mg/dL were considered diabetic and further included in the study. The diabetic rats were divided into the diabetic control and the treatment group. A total of 24 animals (n = 8 in each group) were used in all the studied groups. Treatment group rats were given p. o. a bioflavonoid combination of soy extract (300 mg/kg) [13], ginger extract (75 mg/kg) [14], and hesperetin (100 mg/kg) [15] dispersed in distilled water for the tested period of twenty-four weeks.

2.3. Collection of samples

Urine was collected over 24 h using metabolic cages, yielding 3–5 mL of urine. Rats were euthanized using a saturated CO_2 chamber. Blood samples (2–3 mL) were obtained via cardiac puncture, and targeted tissues were collected by surgical procedure at the study's end.

2.4. Determination of biochemical parameters

A strip-based glucose monitoring system (Gluco One, Dr. Morepen, South Korea) was used to estimate the random blood glucose by pricking the rat's tail. According to the manufacturer's guidelines (Abbkine, China), serum insulin levels were assessed using ELISA. The homeostasis model assessment (HOMA-IR) approach evaluated insulin resistance status using the formula HOMA = fasting glucose (mg/dl) × fasting insulin (μU/ml)/405.

2.5. Oral glucose tolerance test (OGTT) and area under the curve (AUC)

Overnight fasted rats were used for the study. Zero-hour fasting blood glucose levels were determined as mentioned above. Following the reading, the rats received glucose orally at a 2.0 g/kg body weight. After administering glucose, blood sugar levels were checked at 30, 60, and 120 min. The areas under the curve (AUC) for the OGTT were computed using the linear trapezoidal approach.

2.6. Serum and urine parameters

At the end of twenty-four weeks, blood urea nitrogen (BUN), serum, and urinary creatinine (Scr) were measured using an automatic biochemical analyzer (Quickem 160, Quicklab, India). Urinary albumin was measured using ELISA kits (Elabsciences, USA). The urine albumin excretion rate (UAER) was consequently determined.

2.7. TGF-β and total antioxidant capacity

Per the manufacturer's instructions, TGF-β was measured using an ELISA kit (Elabsciences, USA) [16]. The total antioxidant capacity of the kidney homogenate (10 μL of the sample) was measured by a colorimetric technique using a commercial kit (Cayman, USA) [17].

2.8. Gel zymography

In 1× ice-cold PBS, all tissue samples were homogenized. The supernatant was separated, and Bradford's technique calculated the total protein content. The samples (25 μg/mL of total protein) were electrophoretically run on a 7.5 % acrylamide gel with 0.1 % gelatin. The gel underwent two washings with 2.5 % Triton X to renature the proteins. A 0.05 M Tris solution containing ten mM $CaCl_2$ and NaCl incubated the gel. Coomassie blue was then used to dye the gel. Gel pictures were captured using the Chemic Doc MP imaging device (Bio-Rad Lab, USA) and Image Lab software version 5.0.

2.9. Western blot analysis

Protein was isolated from the kidney tissues via homogenization and further processing through the total protein extraction kit (Real Gene Labs, India) per the manufacturer's instructions. A bicinchoninic acid protein estimation kit (G-biosciences, USA) was used for protein concentration measurement. The proteins were separated on SDS polyacrylamide gel (10 % resolving gel) and immunoblotted on a PVDF membrane. Anti-MMP-9, TGF-β, TIMP-1, GLUT-4, and anti-β-actin (Sigma-Aldrich, USA) were the primary antibodies. The secondary antibodies were HRP-conjugated, and an enhanced chemiluminescence (ECL) technique was used for the band detection (Bio-Rad Lab, USA). The images were captured via an E-Gel imager (Tanon5200Multi, Shanghai, China).

2.10. Reverse transcription-polymerase chain reaction

Fresh kidney tissues were dissected from the rats in all the groups. Total RNA extraction was done using Trizol reagent (Life

Table 1
List of primers used for the mRNA expression.

Gene	Forward primer	Reverse primer
TGF-β	GACCGCAACAACGCAATCTA	CAGGTGTTGAGCCCTTTCCA
MMP-9	CACTCCTACTCTGCCTGCAC	AAACAGGCTGTACCCTTGGT
TIMP-1	TGCTCAAAGGATTCGACGCT	GGGATGGCTGAACAGGGAAA
GLUT-4	ATTCTGGTTGCCCAGGTGTT	CTCAGCCAGTGCATCAGACA
β-actin	CTCTGAACCCTAAGGCCAACC	CACAGGATTCCATACCCAGGAA

Technologies Inc., USA). The quality of RNA was assessed using a nanophotometer instrument (NP80, Implen, Munich, Germany) with a reading at 260/280 nm. RNA was converted into cDNA using a verso cDNA synthesis kit (Thermo Fischer, USA) following the manufacturer's protocol. The PCR reaction was conducted on a VeritiPro thermal cycler (Applied Biosystems, USA). The list of primers used in the study is mentioned in Table 1. The results were evaluated using densitometric analysis on the PCR products on agarose gel.

2.11. Immunohistochemical (IHC) analysis

Yang and the team's protocol was used to perform the IHC [18]. Briefly, slices of kidney tissue (3 mm thick) fixed in paraffin were deparaffinized. The slides were then exposed to 3 % hydrogen peroxide (in methanol) for 10 min. Citrate buffer (pH 6.0) was used to perform antigen retrieval for 5 min in a water bath at 100 °C. Afterward, the slides were treated with 10 % goat serum for 10 min at room temperature. The slides were incubated overnight at 4°C with anti-MMP-9 and anti-TGF-β antibodies (1:500, Santa Cruz, CA, USA). The slides were then washed three times. The slides then spent another 30 min with horseradish peroxidase (HRP). Following secondary antibody incubation, peroxidase activity sites were identified using the substrate 3,3-diamino-benzidine tetrahydrochloride (DAB). Hematoxylin was used as a counterstain.

2.12. Statistical analysis

The data were presented as Mean ± SEM. Statistical analysis was conducted using one-way analysis of variance (one-way ANOVA) followed by an appropriate post-hoc test unless otherwise stated. Significance was determined at $p < 0.01$ for all analyses. GraphPad Prism (ver. 8) was utilized for statistical analysis.

3. Results

3.1. Effect of bioflavonoid combination on body weight

The diabetic group exhibited body weights similar to the control group, with no significant difference observed. Bioflavonoid intervention also did not significantly change the body weight of the rats (Fig. 1). The body weight at the start of the study was 80–90 g in each group, and it was 170–180 g at the end of the study.

3.2. Effect of bioflavonoid combination on glycemic status

After twenty-four weeks of the study period, fasting blood glucose levels exhibited a notable increase ($p < 0.001$) in the diabetic control group compared to the control group. Additionally, the diabetic control group showed significantly lower serum insulin levels and higher HOMA-IR values than the control group. Treatment with the bioflavonoid combination for twenty-four weeks resulted in a significant reduction ($p < 0.001$) in fasting blood glucose levels compared to the diabetic control group (Fig. 2). Moreover, serum insulin levels were observed to increase while HOMA-IR values decreased in the treatment group compared to the diabetic control group (Fig. 3).

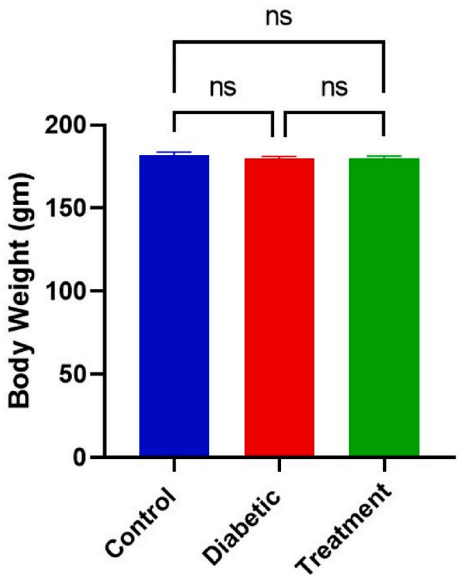

Fig. 1. Effect of bioflavonoid combination on body weight. Data were presented as mean ± SEM (n = 8; each group). *ns – non-significant.

3.3. Effect of bioflavonoid combination interventions on OGTT

OGTT in the diabetic control group exhibited significantly higher glucose concentrations at all the tested time points compared to normal rats (Fig. 4A). Treatment with a bioflavonoid combination significantly lowered the glucose concentration compared to the diabetic rats. The area under the curve values for the diabetic control group also showed significantly higher values ($p < 0.001$) compared to the normal rats (Fig. 4B). Treatment with a bioflavonoid combination significantly decreased the area under cure as compared to the diabetic rats.

3.4. Effect of bioflavonoid combination on serum and urine parameters related to diabetic nephropathy

We assessed various parameters, including blood urea nitrogen (BUN), serum creatinine, serum albumin, urinary albumin, urine creatinine, creatinine clearance, and urinary albumin excretion rate (UAER) across all groups. Diabetic rats exhibited notably higher BUN levels than normal rats, with statistical significance ($p < 0.001$). However, treatment with the bioflavonoid combination led to a significant ($p < 0.05$) reduction in BUN levels in the treated group compared to the diabetic control (Fig. 5).

3.5. Serum creatinine and albumin

Serum creatinine levels of diabetic rats were significantly higher ($p < 0.001$) compared to normal rats. Administration of a bioflavonoid combination significantly ($p < 0.05$) reduced serum creatinine levels as compared to the diabetic control (Fig. 6A). Serum albumin levels in the diabetic group were found to be significantly ($p < 0.001$) low compared with the control. The bioflavonoid combination significantly ($p < 0.05$) raised the level of serum albumin (Fig. 6B).

3.6. Effect of bioflavonoid combination on urinary parameters

Urinary albumin and urinary albumin-to-creatinine ratio (UAER) were notably elevated ($p < 0.001$) in the diabetic control group compared to the normal control rats. However, treatment with the bioflavonoid combination led to a significant reduction ($p < 0.05$) in both urinary albumin and UAER values. Conversely, urinary creatinine and creatinine clearance were significantly diminished ($p < 0.001$) in the diabetic control group compared to the control group. Administration of the bioflavonoid combination resulted in a significant increase ($p < 0.05$) in both urinary creatinine and creatinine clearance (Fig. 7).

3.7. Effect of bioflavonoid combination on TGF-β levels in kidney

The level of TGF- β estimated through ELISA showed higher values in the diabetic rats than in the normal rats. However, the treatment with a bioflavonoid combination significantly decreased the TGF- β in the treated group compared to the diabetic group (Fig. 8).

3.8. Effect of bioflavonoid combination on total antioxidant capacity (TAC)

Diabetic control rats significantly declined TAC levels compared to the control group ($p < 0.001$). However, treatment with a bioflavonoid combination significantly ($p < 0.05$) increased kidney TAC levels as compared to the diabetic control animals (Fig. 9).

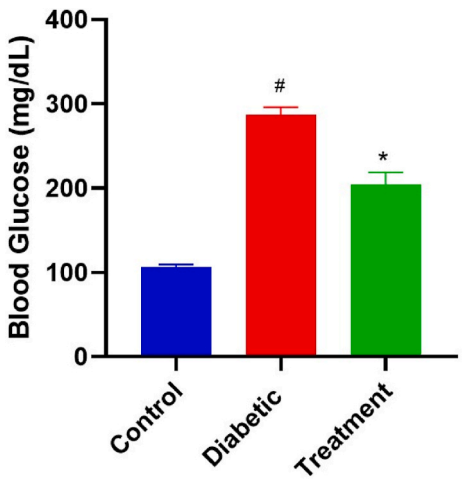

Fig. 2. Effect of Bioflavonoid combination on blood glucose. Data were presented as mean ± SEM (n = 8; each group). #$p < 0.001$ vs normal *$p < 0.001$ vs. Diabetic control.

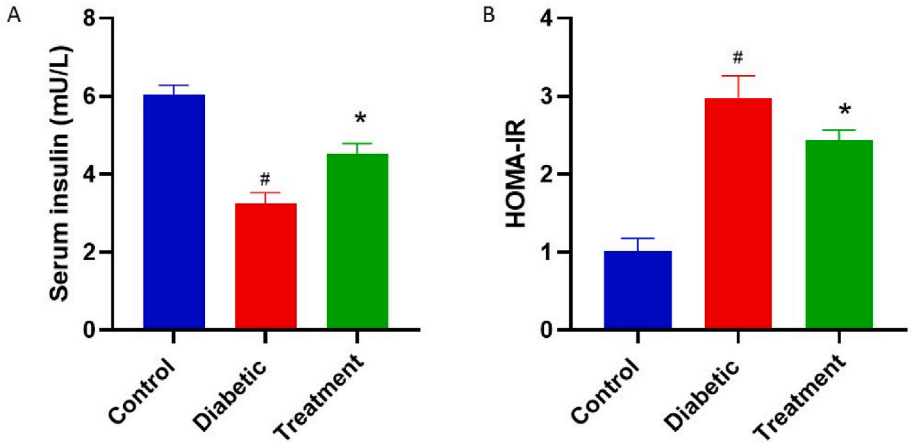

Fig. 3. Effect of Bioflavonoid combination on A. Serum insulin and B. HOMA IR. Data were presented as mean ± SEM (n = 8; each group). #p < 0.001 vs. control *p < 0.001 vs. Diabetic control, One Way Analysis of Variance (Tukey's Multiple Comparison).

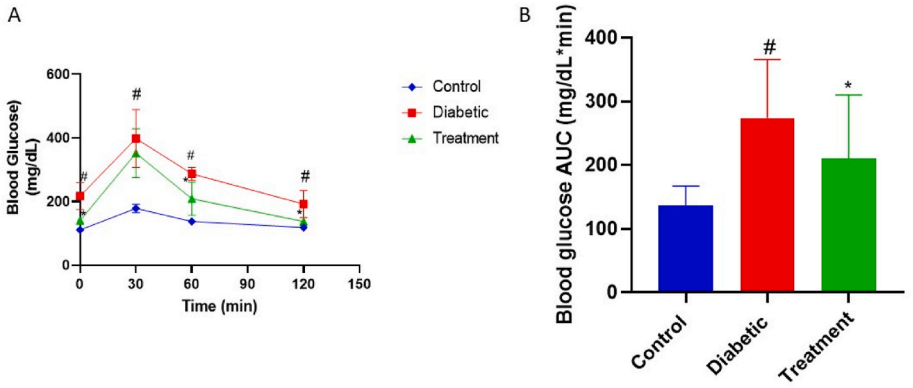

Fig. 4. A. Effect of bioflavonoid combination on parameters of oral glucose tolerance test (OGTT). A. Concentration vs time plot. Each value represents mean ± SEM (n = 8; each group). #p < 0.001 vs. control, *p < 0.05 vs. diabetic control, One-way ANOVA (Dunnett's multiple comparisons). B. AUC calculation for the blood glucose. Values are represented as mean ± SEM (n = 8; each group). #p < 0.001 vs. control, *p < 0.001 vs. Diabetic, One-way ANOVA (Dunnett's multiple comparison).

3.9. Effect of bioflavonoid combination on gelatinase activity and tissue expression of MMP-9

The gel zymography data showed that the diabetic control kidney samples had notably high expression of MMP-9. The bioflavonoid combination reduced the expression of MMP-9 in the kidney tissue. IHC findings showed relatively higher expression of MMP-9 in the renal tubules of the diabetic rats than in the regular control group. The rats with the bioflavonoid combination showed a marked reduction in the expression of MMP-9 (Fig. 10).

3.10. Effect of bioflavonoid combination on tissue expression of TGF-β

TGF-β is an essential inflammatory marker of diabetic nephropathy, leading to glomerulosclerosis and interstitial fibrosis. Here, we have performed immunohistochemistry for TGF-β to evaluate its expression in all three groups. It was found that there was an increased expression of TGF- β in diabetic kidney samples as compared to the treatment group (Fig. 11).

3.11. Effect of bioflavonoid combination on protein expression of TGF-β, GLUT-4, MMP-9 and TIMP-1

The diabetic control group showed significantly increased expression of MMP-9 and TGF-β, whereas TIMP-1 and GLUT-4 expression was downregulated. However, the bioflavonoid combination significantly increased the expression of TIMP-1 and GLUT-4 and downregulated the expression of MMP-9 and TGF- β (Fig. 12).

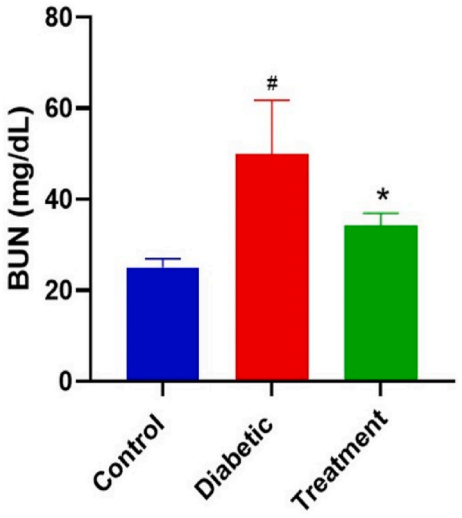

Fig. 5. Effect of Bioflavonoid combination on BUN level. Each value is expressed as mean ± SEM (n = 8; per group). # denotes $p < 0.001$ compared to the control, while * indicates $p < 0.05$ compared to the diabetic control. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test.

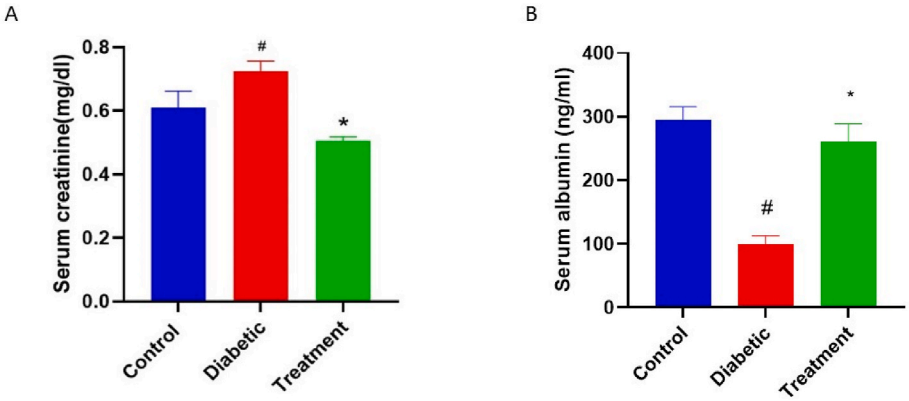

Fig. 6. The effect of bioflavonoid combination on A. Serum creatinine and B. Serum albumin. Each value represents mean ± SEM (n = 8; each group). #$p < 0.001$ vs. control,*$p < 0.05$ vs. diabetic control, One-way ANOVA (Tukey's multiple comparison).

3.12. Effect of bioflavonoid combination on gene expression of TGF-β, GLUT-4, MMP-9, and TIMP-1

mRNA expression of GLUT-4 and TIMP-1 were significantly downregulated in the diabetic kidney tissue. Whereas MMP-9 and TGF-β were found to be upregulated. Treatment with a bioflavonoid combination significantly upregulated mRNA expression of GLUT-4 and TIMP-1, while TGF- β and MMP-9 were significantly downregulated (Fig. 13).

4. Discussion

Diabetic nephropathy (DN) is a microvascular complication of uncontrolled blood sugar levels. It is the leading cause of end-stage renal disease (ESRD) worldwide [19]. Although strict glycemic control has been shown to hasten the progression of DN pathogenesis, it fails to provide complete remission [20]. Though antidiabetic drugs effectively reduce blood glucose, they cannot target multiple pathways involved in the DN progression.

Plant-based secondary metabolites have been shown to have promising benefits in diseases such as diabetes, hypertension, and obesity [21–23]. These secondary metabolites have been proven to target multiple pathways synchronously [24–26]. The current study has selected plant-based bioflavonoid combinations systematically evaluated individually at our laboratory through various disease models [13–15].

The current study exhibited DN development in rats, represented by significantly elevated biochemical parameters such as blood urea nitrogen (BUN) and serum creatinine (sCR) in the diabetic group. Elevated BUN is an excellent clinical marker of renal

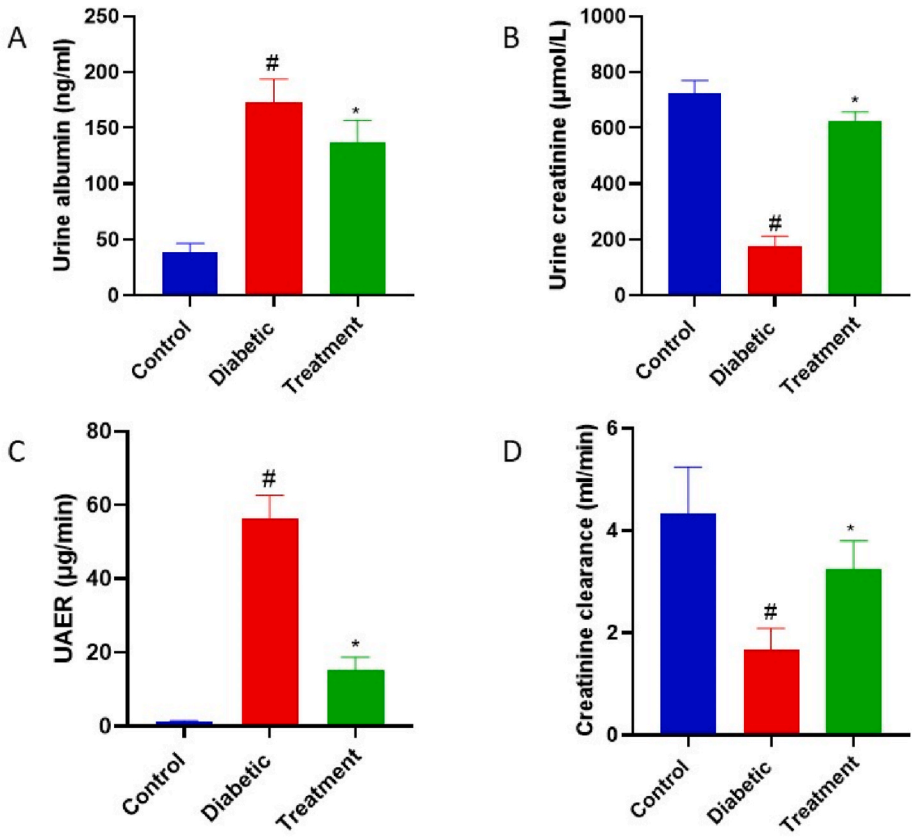

Fig. 7. Effect of Bioflavonoid combination on A. urine albumin B. urine creatinine C. UAER and D. Creatinine clearance. Each value represents mean ± SEM (n = 8; each group). #p < 0.001 vs. control,*p < 0.001 vs. diabetic control, One-way ANOVA (Tukey's multiple comparisons).

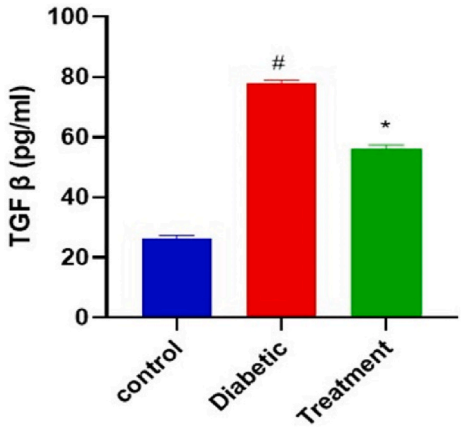

Fig. 8. Effect of Bioflavonoid combination on TGF β level. Each value represents mean ± SEM (n = 8; each group). #p < 0.001 vs. control,*p < 0.001 vs. diabetic control, One-way ANOVA (Tukey's Multiple Comparison).

impairment. Moreover, elevated BUN is associated with the catabolic status of the body [27,28]. Besides, increased catabolism suggests an increase in reactive oxygen species (ROS). The selected bioflavonoids have proven antioxidant activity. However, it has been reported that the administration of hesperetin attenuates hyperglycemia and dyslipidemia through ameliorating antioxidant competence in STZ-induced experimental rats [29]. It has been shown that certain varieties of soybeans, particularly in their seed coat or hulls, possess antioxidant properties, offering potential health benefits. Numerous studies have investigated soybean seeds' phenolic composition and antioxidant properties and their by-products, such as soy milk, tofu, and fermented products. These investigations aim to explore their potential in preventing various diseases, including certain cancers, osteoporosis, chronic renal disease, diabetes,

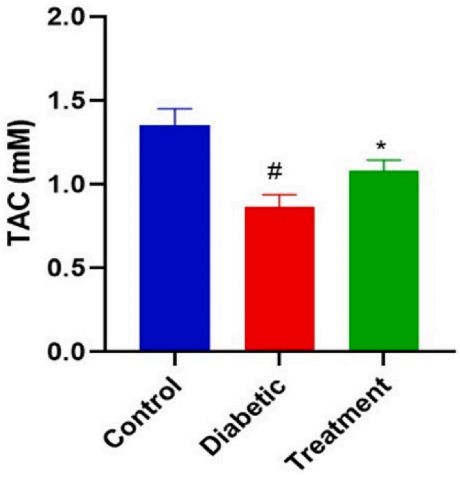

Fig. 9. Effect of bioflavonoid combination treatment on kidney TAC. Values are represented as mean ± SEM (n = 8; each group).#p < 0.001 vs. normal control, *p < 0.05 vs. Diabetic control, One Way Analysis of Variance (Tukey's Multiple Comparison).

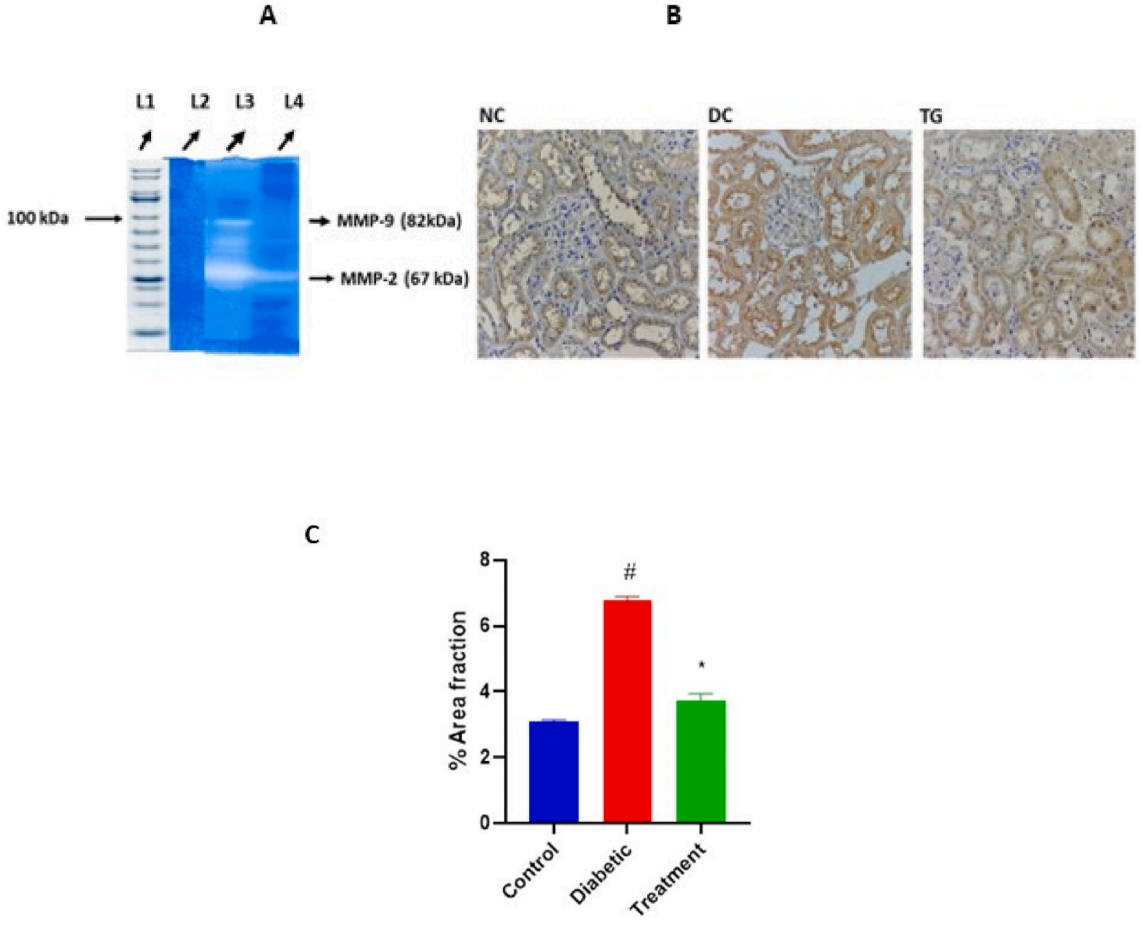

Fig. 10. A. Gel zymogram showing the intense MMP-9 activity in the diabetic kidney (L1-ladder, L2-normal control, L3-diabetic control, L4-treatment group). **B**. Immunohistochemical staining of kidney sections at the end of twenty-four weeks in each group of rats. NC (normal control) shows weak cytoplasmic positivity of MMP-9, DC (diabetic control) shows strong membranous and granular cytoplasmic positivity of MMP-9, while TG (treatment group) shows weak membranous expression of MMP-9. X400. **C**. semi-quantitative bar graphs for MMP-9. Image-J software was used for the semi-quantitative analysis. #p < 0.001 vs. control, *p < 0.05 vs. Diabetic control, One-way ANOVA (Tukey's Multiple Comparison).

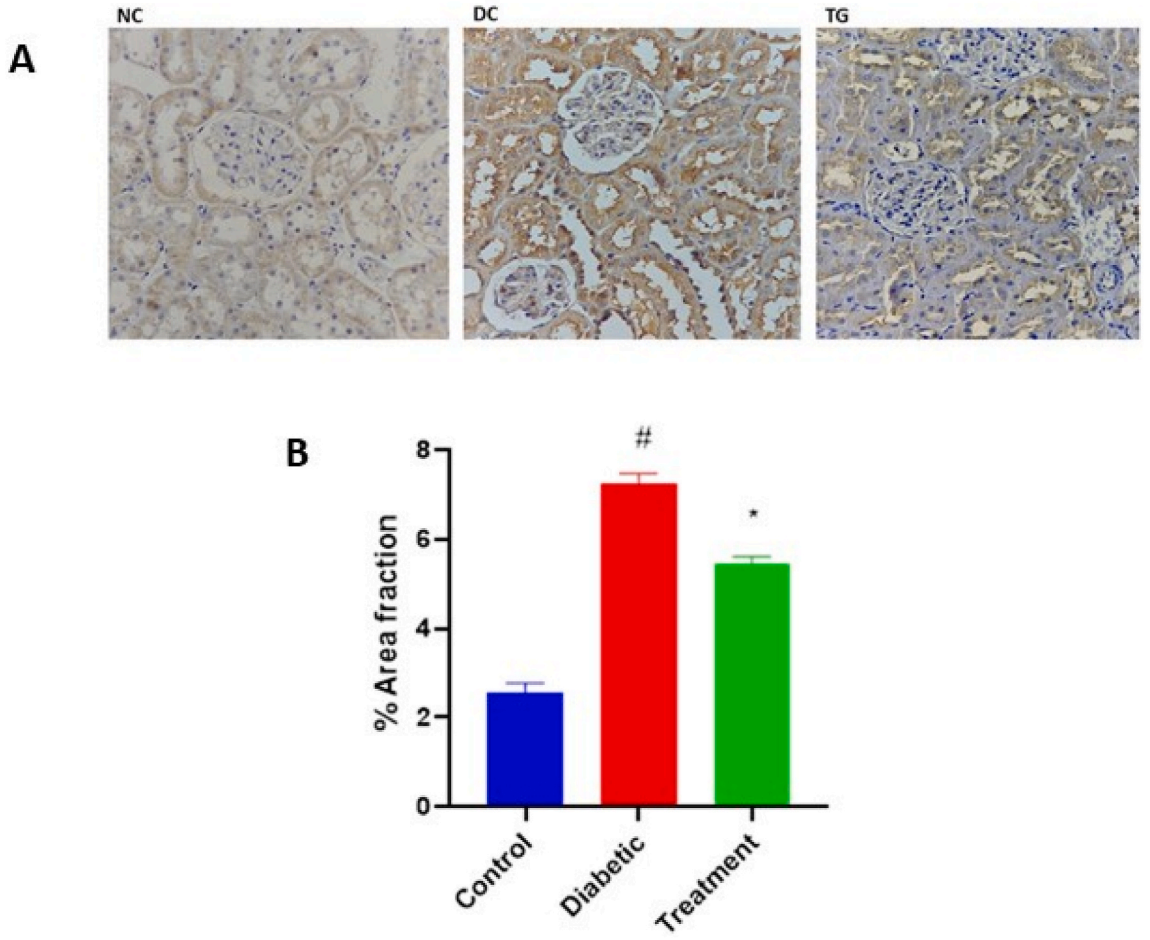

Fig. 11. A. Immunohistochemical staining of TGF-β in kidney sections. (NC-normal control, DC-diabetic control, TG-treatment group). Strong membranous and granular cytoplasmic positivity of TGF-β in DC, whereas weak reactivity of TGF-β in NC and TG groups. X400. **B** semi-quantitative bar graphs for TGF-β. Image-J software was used for the semi-quantitative analysis. #$p < 0.001$ vs. control, *$p < 0.05$ vs. Diabetic control, One-way ANOVA (Tukey's Multiple Comparison).

and coronary heart disease, and their role in combating atherosclerosis [30].

Moreover, ginger is a common spice incorporated into diverse culinary traditions globally. Its phytochemical makeup highlights its potential health benefits. As an antioxidant, ginger shields the body from oxidative stress and DNA harm by combating free radicals. Moreover, its therapeutic impact on diabetic complications stems from its ability to diminish oxidative stress and inflammation, in part through inhibiting the NF-κB signaling pathway [31]. In our investigation, we also observed a reduction in the total antioxidant capacity (TAC) in the diabetic group compared to the control.

The current study exhibited the development of diabetes by showing higher blood glucose and decreased insulin levels. It increased HOMA-IR scores and area under the curve in the oral glucose tolerance test (OGTT). Although our bioflavonoid combination effectively reduced the hyperglycemic condition, the effect was marginal (~25 % and ~15 % reduction in blood glucose and HOMA-IR score, respectively). This data exhibits the flavonoid combination's nephropathy protective potential in an independent glycemic control manner.

Matrix metalloproteases (MMPs) are crucial extracellular matrix (ECM) modulation enzymes. Specifically, MMP-9, a gelatinous class of MMP, has been reported to induce podocyte dedifferentiation and tubular damage in diabetic animals [32]. In the present study, gel zymography showed the upregulation of MMP-9 in the kidneys of diabetic rats. Through the immunohistochemistry (IHC) technique, this study confirmed the overexpression of MMP-9 in the tubular cells of diabetic kidneys. Additionally, significantly increased urinary albumin in diabetic rats confirmed podocyte cell-cell junction loss. The bioflavonoid combination reduced MMP-9 expression in both gel zymography and IHC. These results were further substantiated by Western blot and PCR-based experiments.

Transforming growth factor β (TGF- β) has been considered as one of the potential modulators of renal fibrosis. TGF-β has been postulated to stimulate epithelial-mesenchymal transition (EMT) in the kidney [33]. Interestingly, our bioflavonoid combination attenuated the TGF- β expression. Our data aligns with the findings of other groups in DN animals where crosstalk between TGF- β and MMP-9 was shown [34].

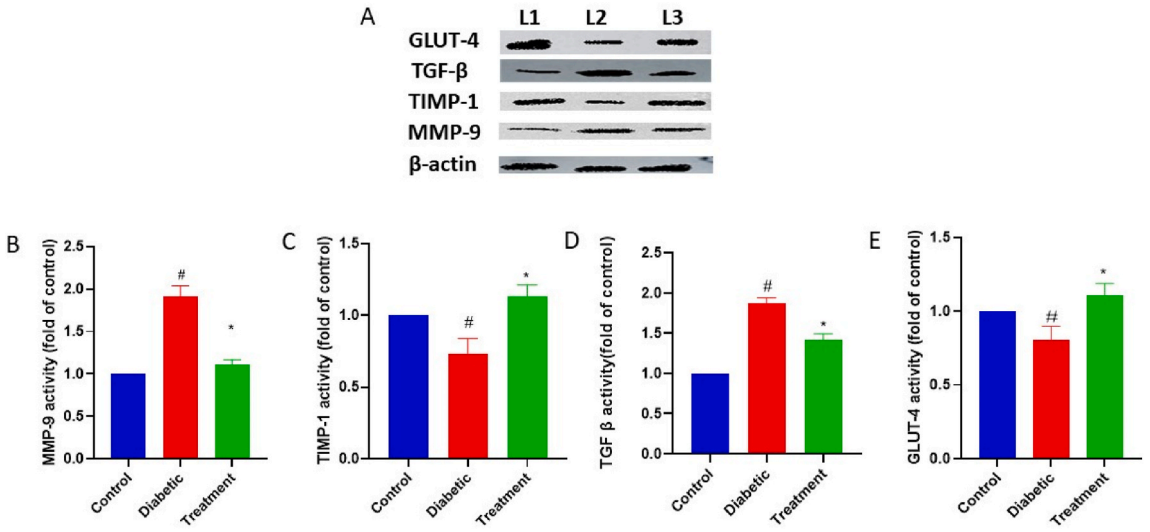

Fig. 12. Representative images of A. Western blot for various proteins and semi-quantitative bar graphs for B.MMP-9, C. TIMP-1, D. TGF-β, and E. GLUT-4. Image-J software was used for the semi-quantitative analysis of protein expression. B-actin was used as a housekeeping protein. #$p < 0.001$ vs. control, *$p < 0.05$ vs. Diabetic control, One-way ANOVA (Tukey's Multiple Comparison).

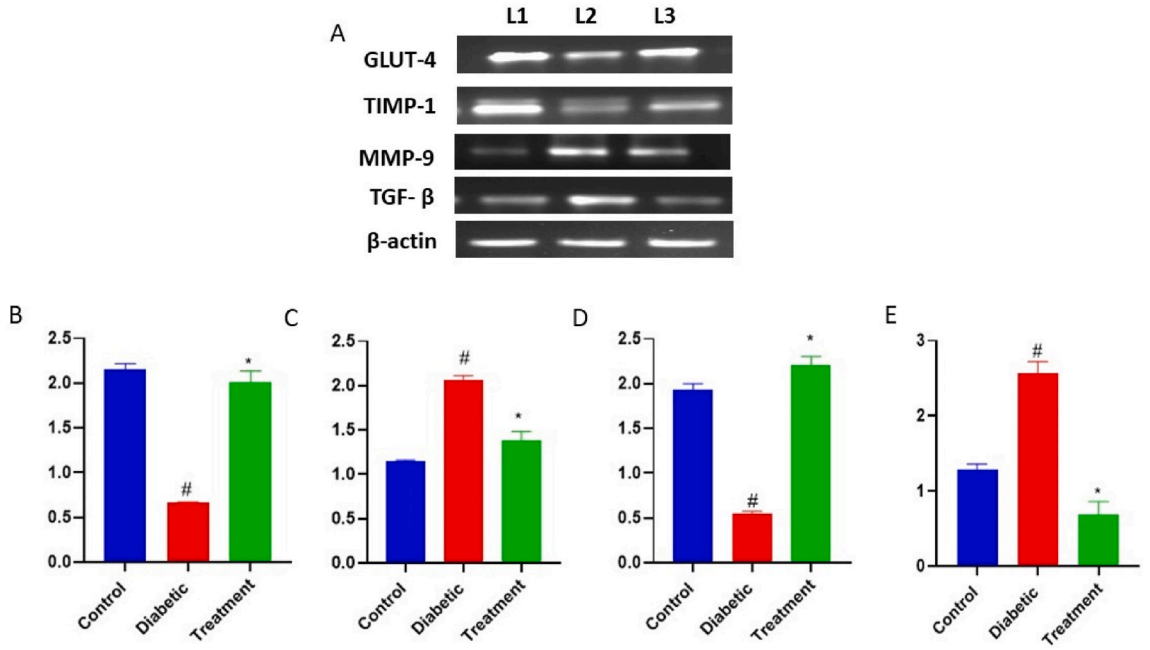

Fig. 13. A. PCR gel image showing mRNA expression of various genes; semi-quantitative analysis of B. GLUT-4, C. MMP-9, TIMP-1, and E. TGF-β. Image-J software was used for the semi-quantitative analysis of mRNA expression. β-actin was used as a housekeeping gene. #$p < 0.001$ vs. control, *$p < 0.05$ vs. Diabetic control, One-way ANOVA (Tukey's Multiple Comparison).

The proteolytic activity of MMP-9 has been reported to be balanced by the Tissue inhibitor of metalloproteases-1 (TIMP-1). The imbalance of TIMP-1 and MMP-9 has been associated with various diabetic complications [35,36]. Our study exhibited down-regulation of TIMP-1 in Western blot and PRC experiments, contrary to MMP-9, which was upregulated in the diabetic control group. The bioflavonoid combination treatment group showed upregulation of TIMP-1 in the kidney tissue.

Interestingly, our study also showed downregulation of GLUT-4 transporter in the diabetic group. The hyperglycemia-mediated downregulation of GLUT-4 has been shown by several groups [37]. However, Guzman and the team showed nutrient sensing as a protective phenomenon of renal cells in GLUT-4 downregulation [38]. We postulate that the GLUT-4 downregulation in the diabetic group in our study could be defending renal cells from damage. The bioflavonoid combination mediated upregulation of GLUT-4 might

indicate a change in the renal cell environment from hostile to near normal.

The bioflavonoid combination showed marginal glycemic control in the neonatal diabetic model. However, other cardinal parameters of DN were significantly modulated with the bioflavonoid combination.

5. Conclusions

In this study, diabetic nephropathy, a severe diabetes complication, was addressed by testing a bioflavonoid combination of ginger, soy extracts, and hesperetin on diabetic rats. The treatment significantly reduced hyperglycemia and improved urinary parameters, suggesting improved renal function. Notably, it modulated MMP-9/TIMP-1 ratios, upregulated GLUT-4, and downregulated TGF-β expression, enhancing total antioxidant capacity. These multifaceted improvements indicate that the bioflavonoid combination holds promise as a therapeutic strategy for diabetic nephropathy, marking a significant step forward in managing this condition with no current dedicated treatments.

Ethical approval

The study protocol was approved by the standing Institutional Animal Ethics Committee (/IAEC/2020/II-R02). All experimental methodologies and animal handling techniques followed the CPCSEA regulations.

CRediT authorship contribution statement

Ritu: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Yifan Xiong:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Hanuman Prasad Sharma:** Writing – review & editing, Formal analysis. **Ramesh K. Goyal:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization. **Sonia Narwal:** Writing – review & editing, Formal analysis. **Ajay Berwal:** Writing – review & editing, Formal analysis. **Sourabh Jain:** Writing – review & editing, Formal analysis. **Meher Priya:** Writing – review & editing, Formal analysis. **Manisha Singh:** Writing – review & editing, Formal analysis. **Gaurav Agarwal:** Writing – review & editing, Formal analysis. **Xiaoyan Wang:** Writing – review & editing, Supervision, Resources, Formal analysis, Conceptualization. **Bairong Shen:** Writing – review & editing, Formal analysis. **Rajeev K. Singla:** Writing – review & editing, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We acknowledge the Indian Council of Medical Research (ICMR), India, for Providing a Senior Research Fellowship to Ms. Ritu.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33217.

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