Journal of Research in Pharmacy

Research Article

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Polyphenol harmony: Ferulic acid and protocatechuic acid combination attenuates diabetic nephropathy in Sprague-Dawley rats

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Received: 26 August 2023 / Revised: 20 December 2023 / Accepted: 21 December 2023

ABSTRACT: Diabetic nephropathy (DN) is an alarming consequence of diabetes mellitus, characterized by progressive kidney dysfunction and damage. Despite advancements in diabetes management, the prevalence of DN remains a significant concern. Natural compounds such as ferulic acid (FA) and protocatechuic acid (PCA) have shown potential in ameliorating diabetic complications. This research paper investigates the individual and combined effects of FA and PCA in an experimental model of DN in rats. Male rats were assigned into five groups: Control, Diabetic control, FA-treated (100 mg/kg, p.o.), PCA-treated (100 mg/kg, p.o.), and FA + PCA-treated groups. Diabetes was induced by streptozotocin injection (55 mg/kg, i.p.), and the treatment groups received oral administration of FA, PCA, or their combination for eight weeks. Renal function, oxidative stress markers, and histopathological changes were assessed at the end of the study.

The diabetic control group exhibited impaired renal function, increased oxidative stress, and histological abnormalities in the kidney tissues. Treatment with FA, PCA, and FA + PCA significantly improved renal function, as evidenced by reduced serum creatinine, cystatin C, blood urea nitrogen, and urine albumin levels. Moreover, these treatments led to a decline in oxidative stress markers and restoration of antioxidant enzyme activities. The combination of FA and PCA showed a synergistic effect, demonstrating a more pronounced improvement in renal parameters and histological changes compared to individual treatments. These findings highlight the potential renoprotective effects of FA and PCA in diabetic nephropathy.

KEYWORDS: Diabetic nephropathy; ferulic acid; protocatechuic acid; streptozotocin; renal function; oxidative stress.

1. INTRODUCTION

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD) worldwide, affecting approximately one-third of individuals with diabetes mellitus. The prevalence of diabetes has been steadily increasing, making DN a major public health concern [1]. According to current statistics, diabetes affects more than 537 million people globally, and it is estimated that around 20-40% of diabetic patients will develop nephropathy during their lifetime. This alarming rise in DN cases necessitates effective therapeutic approaches to manage and prevent the progression of this debilitating condition [2,3].

The pathophysiology of diabetic nephropathy is complex and involves various interrelated mechanisms. Chronic hyperglycemia is the primary driver of DN, leading to the activation of multiple pathways that contribute to kidney damage. Hyperglycemia-triggered oxidative stress is important in the pathogenesis of DN since it contributes to the production of reactive oxygen species (ROS), which cause cellular injury and stimulate inflammatory responses. Additionally, the renin-angiotensin-aldosterone system (RAAS) activation, advanced glycation end-products (AGEs) formation, and abnormal activation of growth factors, like transforming growth factor-beta (TGF-β), further contribute to renal injury and fibrosis [4-6].

Currently, the management of diabetic nephropathy primarily involves strict glycemic control, blood pressure management, and the use of RAAS inhibitors. While these approaches have shown some benefits in slowing the progression of DN, they do not provide complete protection against kidney damage, and the development of ESRD remains a significant concern [7]. Thus, there is a need for novel therapeutic interventions to complement existing treatment strategies.

How to cite this article: Mahajan M, Pandit S, Upaganlwar A, Upasani C. Polyphenol harmony: Ferulic acid and protocatechuic acid combination attenuates diabetic nephropathy in Sprague-Dawley rats. J Res Pharm. 2024; 28(5): 1492-1500.

http://dx.doi.org/10.29228/jrp.827

ISSN: 2630-6344

The limited success of current treatment options highlights the urgent need for new approaches to manage diabetic nephropathy effectively. Novel therapies that can target multiple pathological pathways, including oxidative stress and inflammation, are required to provide comprehensive renal protection. Antioxidants have emerged as potential candidates due to their ability to scavenge ROS and mitigate oxidative stress-induced damage [8]. Ferulic acid (FA) and protocatechuic acid (PCA), natural compounds found in various fruits and vegetables, have demonstrated potent antioxidant and anti-inflammatory properties [9, 10]. Hence, exploring the renoprotective effects of these antioxidants in DN may pave the way for new and effective therapeutic interventions.

In this research, we investigated the individual and combined effects of ferulic acid (FA) and protocatechuic acid (PCA) in an experimental model of diabetic nephropathy in rats. FA and PCA were chosen for their strong antioxidant properties, which are expected to counteract oxidative stress-induced damage in the kidneys. We aimed to evaluate their potential in ameliorating renal dysfunction, reducing oxidative stress, and mitigating histopathological changes associated with DN. The combination of these antioxidants was hypothesized to have a synergistic effect, providing enhanced renoprotection compared to their individual administration.

By studying the effects of FA and PCA in DN, we hope to contribute valuable insights into potential novel therapies for managing diabetic nephropathy. These natural antioxidants may serve as promising adjunctive treatments, providing additional benefits to diabetic patients at risk of developing nephropathy and offering hope for improved clinical outcomes in this challenging and prevalent condition.

Derived from natural sources, these compounds have promising safety profiles. Our study aligns with the growing interest in plant-based therapeutics, offering potential translatability to clinical applications.

In essence, we chose ferulic acid and protocatechuic acid for their antioxidant, anti-inflammatory properties, individual renoprotective effects, potential synergy, and natural origin, aiming to uncover novel therapeutic avenues for diabetic nephropathy.

2. RESULTS

2.1 Effect of FA and PCA on Body weight and Kidney Index

The progression of DN was associated with alterations in body weight among the experimental groups. The diabetic group (D) exhibited a gradual decrease in body weight than the control rats (C) ($p<0.05$) throughout the study period (Table 1). This decrease was indicative of the metabolic disturbances and catabolic effects associated with diabetes. Notably, treatment with FA, PCA, and the combination therapy (FA+PCA) led to the significant ($p<0.001$) attenuation of weight loss than the diabetic (D) group.

The kidney weight to body weight (KB) ratio, termed the Kidney Index, was employed as an indicator of renal hypertrophy. In the diabetic group (D), a significant elevation in Kidney Index was observed in comparısıon to the control group (C) ($p<0.001$), indicating the development of renal hypertrophy in response to diabetes-induced hyperglycemia (Table 1). Notably, treatment with FA, PCA, and the combination therapy led to a significant ($p<0.05$) reduction in kidney index compared to the diabetic group. This reduction indicated a potential attenuation of renal hypertrophy, suggesting that the polyphenol treatments might have intervened in the structural alterations associated with DN (Table 1).

Table 1. Effect of FA, PCA and FA+PCA on body weight and kidney to body weight (KB) ratio

Groups	Body weight (g)				KB ratio (mg/g)
	Week 0	Week 4	Week 8	Week 12	
C	226.6±8.18	238.8±10.86	275.5±9.15•*	298.7±7.95•*	3.66± 0.100
D	232.7±12.41	230.1±8.19	219.5±10.32^{a*}	187.7±6.23$^{a•**}$	8.13±0.248^{a**}
FA	229.8±11.36	232.8±10.18	239.7±9.92^{a*}	245.3±6.38^{ab*}	5.37±0.172^{ab*}
PCA	234.1±10.63	230.3±12.92	242.6±8.34^{a*}	248.8±6.70^{ab*}	5.41±0.138^{ab*}
FA+PCA	236.1±9.96	236.2±9.37	252.4±8.63^{a*}	266.5±6.99^{bcd**}	4.52±0.167^{abcd**}

Values are expressed as mean±SEM; n=6, evaluated using One way ANOVA followed by Tukey's multiple comparison test. [a] as C, [b] as compared D, [c]as compared to FA and [d] as compared to PCA, •compared with the same group at 0 week and * $p<0.05$ and ** $p<0.001$.

2.2 Effect of FA and PCA on Blood Glucose Level and HbA1c

The diabetic group (D) had significantly higher blood glucose levels after streptozotocin (STZ) injection than the control group (C) ($p<0.001$). Diabetic group showed consistently high blood glucose throughout the study period. The treatment with ferulic acid (FA) and protocatechuic acid (PCA) individually resulted in significant reductions in blood glucose levels compared to the diabetic group ($p<0.05$). Remarkably, the combination therapy group (FA+PCA) demonstrated the most substantial decrease in blood glucose levels, suggesting a synergistic effect ($p<0.001$). Moreover, HbA1c % were elevated in the diabetic group (D), indicative of prolonged hyperglycemia. Both FA and PCA treatments led to a significant reduction in HbA1c suggesting improved glycemic control (Table 2).

Table 2. Effect of FA, PCA and FA+PCA on blood glucose and HbA1c %

Groups	Blood glucose (mg/dL)				HbA1c %
	Week 0	Week 4	Week 8	Week 12	
C	90.6± 3.87	90.0±3.64	91.4±3.07	91.4± 3.36	4.23±0.052
D	495.6±12.12^{a**}	508.2±10.74^{a**}	516.7±12.61^{a**}	534.8±14.42^{a**}	14.26±0.43^{a**}
FA	491.2±11.28^{a**}	510.4±13.91^{a**}	374.6±12.52^{ab**}	340.2±10.86$^{ab\bullet*}$	8.63±0.25^{ab**}
PCA	488.3±12.14^{a**}	498.3±13.49^{a**}	397.4±11.87^{ab**}	352.5±12.71^{ab*}	9.01±0.28^{ab**}
FA+PCA	494.1±13.61^{a**}	502.6±11.26^{a**}	329.2±14.28$^{ab\bullet**}$	312.3±9.37$^{abcd\bullet**}$	7.03±0.61^{abcd**}

Values are expressed as mean±SEM; n=6, evaluated using One way ANOVA followed by Tukey's multiple comparison test. [a] as C, [b] as compared D, [c]as compared to FA and [d] as compared to PCA, •compared with the same group at 0 week and * $p<0.05$ and ** $p<0.001$.

2.3 Effect of FA and PCA on Urine volume, Serum creatinine, Microalbumin, Cystatin C and BUN

Changes in urine volume were indicative of the renal function alterations observed in the experimental groups. The diabetic group (D) exhibited a significant ($p<0.001$) increase in urine volume than the control group (C), reflecting altered renal handling of fluid and electrolytes due to diabetic nephropathy. Treatment with ferulic acid (FA), protocatechuic acid (PCA), and the combination therapy (FA+PCA) resulted in a significant ($p<0.001$) decrease in urine volume in comparision to the diabetic group (D).

Serum creatinine levels, a marker of kidney function, were elevated significantly ($p<0.001$) in the diabetic group (D) in comparision to the control group. Animals treated with FA, PCA, and the combination therapy led to significant reductions in serum creatinine levels ($p<0.001$), signifying enhanced renal function compared to the untreated diabetic group (D) (Table 3).

Table 3. Effect of FA, PCA and FA+PCA on Serum creatinine and urine volume

Groups	Serum creatinine (mg/dL)				Urine volume (mL/Day)
	Week 0	Week 4	Week 8	Week 12	
C	0.416±0.036	0.414±0.025	0.421±0.051	0.418±0.016	22.3±2.56
D	0.659±0.04^{a*}	1.74±0.29$^{a\bullet**}$	2.63±0.15$^{a\bullet**}$	2.89±0.08$^{a\bullet**}$	86.8±5.76^{a**}
FA	0.646±0.029^{a*}	1.78±0.05$^{a\bullet**}$	1.45±0.038$^{ab\bullet**}$	1.12±0.09$^{ab\bullet**}$	52.3±4.45^{ab**}
PCA	0.648±0.06^{a*}	1.76±0.11$^{a\bullet**}$	1.84±0.05$^{ab\bullet**}$	1.36±0.06$^{ab\bullet**}$	62.7±5.14^{ab**}
FA+PCA	0.651±0.041^{a*}	1.8±0.06a$^{\bullet**}$	1.11±0.067$^{ab\bullet**}$	0.81±0.07$^{abcd\bullet**}$	38.4±4.28^{abcd*}

Values are expressed as mean±SEM; n=6, evaluated using One way ANOVA followed by Tukey's multiple comparison test. [a] as C, [b] as compared D, [c]as compared to FA and [d] as compared to PCA, •compared with the same group at 0 week and * $p<0.05$ and ** $p<0.001$.

Microalbuminuria, a hallmark of diabetic nephropathy, was significantly raised in the diabetic group (D) as compared to the control rats ($p<0.001$). Administration of FA, PCA, and the combination led to significant reductions in microalbuminuria, suggesting attenuation of renal dysfunction (Table 4). Additionally, cystatin C levels were increased in the diabetic group ($p<0.001$). Both FA and PCA treatments demonstrated significant reductions in cystatin C levels, implying improved glomerular filtration.

Blood urea nitrogen (BUN) levels were employed as a marker of kidney function and nitrogen waste accumulation in the bloodstream (Table 4). In the diabetic group (D), BUN were significantly ($p<0.001$) elevated than the control group (C), indicative of impaired renal filtration and clearance. Treatment with FA,

PCA, and the combination therapy resulted in significant reduction in BUN compared to the diabetic group. This reduction suggested a potential enhancement of glomerular filtration and renal excretory function in response to polyphenol treatments (Table 4).

Table 4. Effect of FA, PCA and FA+PCA on microalbumin, cystatin C and BUN

Groups	Microalbumin (mg/L)	Cystatin C (mg/L)	BUN (mg/dL)
C	2.1±0.071	0.52±0.11	16.3±0.62
D	14.8±0.607^{a**}	6.84±0.67^{a**}	42.09±1.06^{a**}
FA	7.63±0.253^{ab**}	3.86±0.23^{ab**}	28.8±0.75^{ab**}
PCA	8.22±0.372^{ab**}	4.07±0.36^{ab*}	31.3±0.96^{ab**}
FA+PCA	4.06±0.218^{abd**}	2.26±0.28^{abcd**}	22.1±0.28^{abd*}

Values are expressed as mean±SEM; n=6, evaluated using One way ANOVA followed by Tukey's multiple comparison test. [a] as C, [b] as compared D, [c]as compared to FA and [d] as compared to PCA. * p<0.05 and ** p<0.001

2.4 Effect of FA and PCA on Antioxidant parameters

The degree of lipid peroxidation and underlying oxidative damage was assessed using malondialdehyde (MDA). The diabetic group (D) exhibited significantly (p<0.001) elevated MDA levels compared to the control group (C), indicating augmented lipid peroxidation due to diabetic nephropathy-induced oxidative stress. Treatment with ferulic acid (FA), protocatechuic acid (PCA), and the combination therapy (FA+PCA) led to significant (p<0.001) reductions in MDA levels as compared to the diabetic group i.e. D. These reductions implied attenuation of oxidative damage to lipids in response to polyphenol treatments (Figure 1).

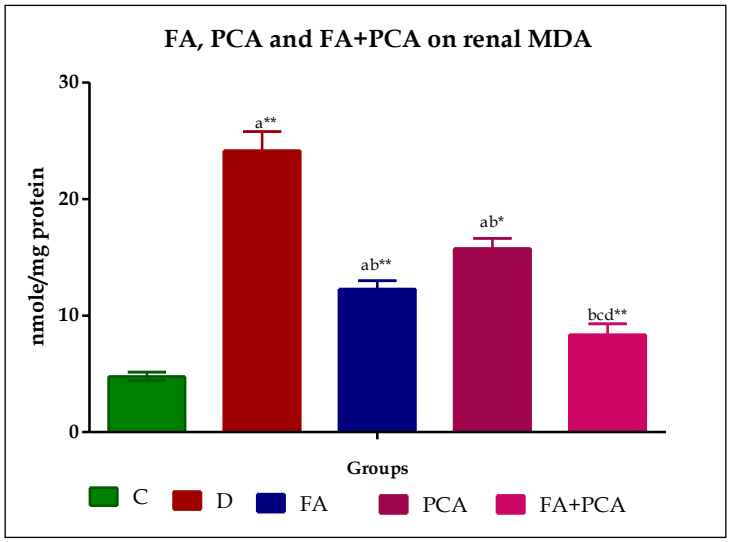

Figure 1. Effect of FA PCA and FA+PCA on MDA level in renal homogenate of diabetic rats.
Values are expressed as mean±SEM; n=6, evaluated using One way ANOVA followed by Tukey's multiple comparison test. [a] as C, [b] as compared D, [c]as compared to FA and [d] as compared to PCA, •compared with the same group at 0 week and * p<0.05 and ** p<0.001.

Superoxide dismutase (SOD) activity, an important antioxidant enzyme, was evaluated to assess the cellular antioxidant defense system. The diabetic group (D) displayed significantly (p<0.001) reduced SOD activity compared to the control group (C), indicating compromised antioxidant defense mechanisms in response to diabetic nephropathy. Treatment with FA, PCA, and the combination therapy led to significant (p<0.001) elevations in SOD activity compared to the diabetic group. These elevations suggested enhancement of cellular antioxidant capacity in the treated groups (Figure 2).

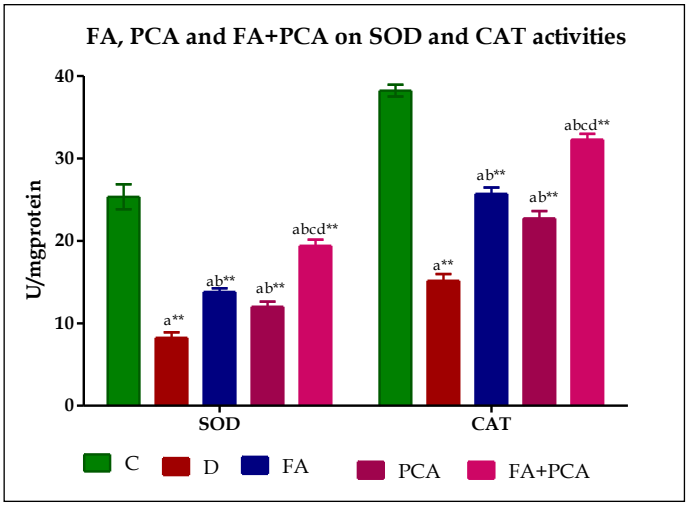

Figure 2. Effect of FA PCA and FA+PCA on SOD & CAT activities in renal homogenate of diabetic rats.
Values are expressed as mean±SEM; n=6, evaluated using One way ANOVA followed by Tukey's multiple comparison test. [a] as C, [b] as compared D, [c]as compared to FA and [d] as compared to PCA. * p<0.05 and ** p<0.001

Catalase (CAT) activity, another key antioxidant enzyme, was examined to evaluate its role in scavenging hydrogen peroxide. The diabetic group (D) exhibited diminished CAT activity compared to the control group (C), suggesting impaired hydrogen peroxide detoxification due to diabetic nephropathy-induced oxidative stress. Treatment with FA, PCA, and the combination therapy led to significant ($p<0.001$) enhancements in CAT activity compared to the diabetic group. These enhancements indicated potential improvements in hydrogen peroxide scavenging and antioxidant defense (Figure 2).

Reduced glutathione (GSH), a critical endogenous antioxidant, was measured to assess its role in counteracting oxidative stress. The diabetic group (D) displayed significantly ($p<0.001$) reduced GSH levels compared to the control group (C), implying GSH depletion due to diabetic nephropathy-associated oxidative burden. Treatment with FA, PCA, and the combination therapy resulted in significant ($p<0.001$) increases in GSH levels compared to the diabetic group. These increases suggested replenishment of GSH stores and a potential boost in antioxidant defense mechanisms (Figure 3).

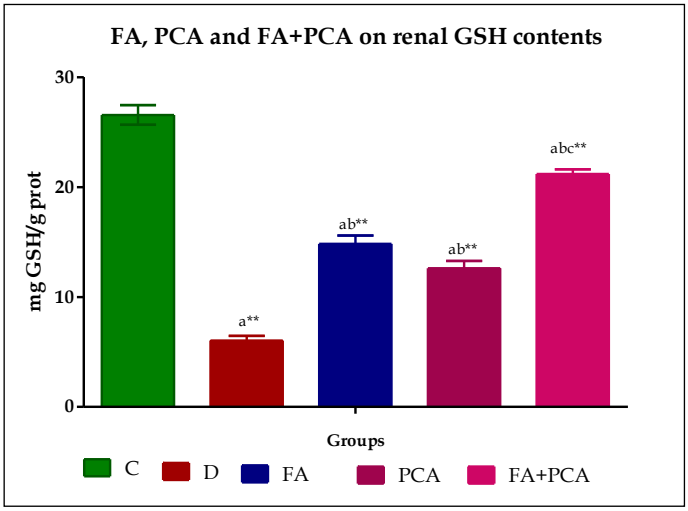

Figure 3. Effect of FA PCA and FA+PCA on GSH content in renal homogenate of diabetic rats.
Values are expressed as mean±SEM; n=6, evaluated using One way ANOVA followed by Tukey's multiple comparison test. [a] as C, [b] as compared D, [c]as compared to FA and [d] as compared to PCA. * p<0.05 and ** p<0.001

2.5 Effect of FA and PCA on Renal histopathology

Histological examination of renal tissues in the diabetic group revealed changes in the glomerular appearance, tubular degeneration, glomerular lobulation, and vacuolar degeneration. Treatment with FA,

PCA, and the combination therapy resulted in notable improvements in renal histopathology. The combination therapy group exhibited the most favorable alterations, including reduced glomerular hypertrophy, diminished tubular dilation, and suppressed interstitial inflammation and fibrosis (Table 5).

Table 5. Effect of FA, PCA and FA+PCA on histopathological changes in the renal tissues

Groups	Intact/normal glomeruli	Normal renal tubules	Glomerular lobulation	Vacuolar degeneration
C	+++	+++	-	-
D	-	-	+++	+++
FA	++	++	-	+
PCA	++	++	+	+
FA+PCA	+++	+++	+	-

+ present & - absent

3. DISCUSSION

Microvascular complications associated with diabetes and related deaths have increased considerably, and are primarily due to a rise in mortality from renal consequences. As a result, DN has emerged as the most prevalent cause of end-stage renal disease (ESRD) globally [11]. Also, chronic hyperglycemia and related oxidative stress aggreviates the progression of DN. This highlights the critical importance of developing and adopting effective antioxidant-based therapies to minimize the impact of diabetic vascular problems [12]. As a result, the study was carried out to investigate the beneficial effects of ferulic acid (FA) and protocatechuic acid (PCA) against experimentally induced DN in rats.

The establishment of the diabetic nephropathy model is crucial for studying the effects of potential therapeutic interventions in a relevant disease context. We monitered fasting blood glucose (FBG, mg/dL), HbA1c and serum creatinine (SCr, mg/dL) in rats to confirm the development of DN. The significant elevation in FBG and Scr at the end of 4th week indicated decline in the kidney function and initiation of nephropathy. This coincides with the prevous studies stating the development of DN at 4th week in rats subjected to single i.p. injection of STZ [13, 14].

The administration of ferulic acid (FA) and protocatechuic acid (PCA) individually and in combination to the diabetic animals for next 8 weeks after confirmation of DN, demonstrated significant renoprotective effects in the experimental DN model. Both antioxidants led to a significant improvement in renal function, as evidenced by marked decrease in Scr and BUN, than the diabetic animals. The decrease in microalbumin and cystatic C levels also pointing the preventive effects of the FA and PCA combination.

The positive effects of FA and PCA in DN are attributed to their potent antioxidant properties [15-18]. The significance of oxidative stress in the pathogenesis of DN is well known and is resulting from excessive reactive oxygen species (ROS) generation [19]. The administration of FA and PCA effectively scavenged ROS and reduced oxidative stress, as indicated by the decline in malondialdehyde (MDA) levels in renal tissues. Additionally, the restoration of superoxide dismutase (SOD), catalase (CAT) activities and reduced glutathione (GSH) concentratıon suggests an enhanced renal antioxidant defense system, protecting the kidneys against oxidative damage.

Histopathological evaluation of kidney tissues further supported the renoprotective effects of FA and PCA. The diabetic control group exhibited glomerular hypertrophy and tubular damage indicative of progressive nephropathy. Administration of FA and PCA individually mitigated these histological abnormalities, with a notable improvement in the histological appearance in the diabetic rat kidneys. However, the most remarkable improvement was observed in the FA + PCA-treated group, suggesting a synergistic effect of the combination in preserving renal architecture.

Both, FA and PCA, used in this study are well known polyphenols. Polyphenols have been shown in studies to have therapeutic value in the treatment of diabetes and its complications. Polyphenols have an array of actions, including reducing oxidative stress by scavenging ROS, diminishing the activity or expression of ROS pathways, and enhancing the expression and activity of antioxidant enzymes [20]. Polyphenols, especially FA and PCA, have been shown in vivo and in vitro to play an important role in the prevention and control of diabetes and its consequences. The mechanisms include but are not limited to, β-cell protection, reducing pancreatic cell apoptosis and promoting proliferation, oxidative stress reduction, insulin signaling activation, inflammation response modification, and inhibition of the formation of advanced glycation end products [21].

The observed polyphenol hormony of FA and PCA in the DN model highlight the potential benefits of combining antioxidants with distinct mechanisms of action. The antioxidative properties of FA and PCA

complement each other, leading to enhanced ROS scavenging and protection against oxidative damage. This synergism may be attributed to their ability to modulate different pathways involved in the pathogenesis of DN, including inflammation and fibrosis.

The promising results obtained from this study provide valuable insights into the potential therapeutic use of FA and PCA in diabetic nephropathy management. Their antioxidative and renoprotective effects make them attractive candidates for future clinical investigations. While this study contributes to our understanding of the possible therapeutic benefits of FA and PCA on diabetic nephropathy, it is important to acknowledge certain limitations. Firstly, our use of a rat model may not perfectly replicate the complex pathophysiology observed in human diabetic nephropathy. Additionally, the study's relatively short duration may not capture long-term effects or complications that could arise over extended periods. Also, it is interesting to study the effect of pretreatment with FA and PCA by starting administration on day 0 rather than waiting for 4 weeks to nephropathy development. Determining the effect of combined therapy of these antioxidants on levels of the inflammatory markers would also be of value and may further confirm the usefulness of FA and PCA in the treatment of diabetic nephropathy.

4. CONCLUSION

In conclusion, the administration of ferulic acid (FA) and protocatechuic acid (PCA), both individually and in combination, demonstrated significant renoprotective effects in the experimentally induced DN. These antioxidants effectively mitigated oxidative stress, improved renal function, and preserved kidney architecture. The synergistic effects observed in the FA + PCA-treated group indicate their potential as complementary therapeutic agents for managing diabetic nephropathy. Further research is warranted to explore their translational potential and to ascertain their clinical benefits in diabetic patients at risk of developing nephropathy.

5. MATERIALS AND METHODS

5.1. Ethical Considerations:

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Shriman Sureshdada Jain College of Pharmacy, Chandwad bearing a reference SSDJ/IAEC/2019-20/01. All the procedures were adhered to the guidelines of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), India for the Care and Use of Laboratory Animals.

5.2. Experimental Animals:

Male Sprague-Dawley rats (n=40) weighing 220-250g were obtained from Lacsmi Biofarms, Pune. The rats were housed in standard laboratory conditions with a controlled temperature of 18-22°C and a 12-hour light-dark cycle. All animals were provided with standard laboratory chow and water ad libitum throughout the study.

5.3 Induction of Diabetes:

Diabetes was induced in the overnight fasted rats by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 55 mg/kg, intraperitoneally. Blood glucose levels were monitored after 72 hours of STZ administration using a glucometer (AlereG1, Korea). Rats with fasting blood glucose levels greater than 280 mg/dL were considered diabetic and included in the study.

The rats were randomly divided into five groups (n=6 per group) as follows:

1. Control group (C): Non-diabetic rats without any treatment.
2. Diabetic control group (D): Diabetic rats without any treatment.
3. FA-treated group (FA): Diabetic rats receiving daily oral administration of FA at a dose of 100 mg/kg [22].
4. PCA-treated group (PCA): Diabetic rats receiving daily oral administration of PCA at a dose of 100 mg/kg [23].
5. FA + PCA-treated group (FA+PCA): Diabetic rats receiving a combination of FA and PCA at their respective doses

FA and PCA were dissolved separately in distilled water before oral dosing to the animals, whereas STZ (55 mg/kg) body weight was prepared freshly by dissolving in cold citrate buffer (pH 4.5) and administered as single intraperitoneal (i.p.) injection.

The treatment with antioxidants were started from week 5 and continued till the end of week 12 for the total of 8 weeks.

5.4 Assessment of Renal Function:

Blood samples from overnight fasted rats were collected via retro-orbital puncture under mild anesthesia at the end of each week from week 1 to week 12 for confirmation of diabetese and DNby assessing FBG (mg/dL) and Scr (mg/dL). At the end of the eight-week treatment period, HbA1c%, SCr, BUN, cystatin C were determined from the blood samples. Urine samples were collected using metabolic cages, and urine volume (mL/day) microalbumin levels were measured.

FBG, Scr and BUN were estimeated in the blood using commercially available biochemical test kits from Arkray Autospan Pvt. Ltd. (Mumbai, India) and Prietest Touch Biochemistry Analyzer, Robonik India Pvt. Ltd. (Mumbai, India). HbA1c, microalbumin and cystatin C were detected by an immunoflurescence kit using i-Chroma II reader (Kin diagnostics, Korea S. No. IR2PK083180). All the reagents were ready to use.

5.5. Measurement of Oxidative Stress Markers:

Following blood collection, rats were euthanized using pentobarbitone sodium and the kidneys were rapidly excised. Renal tissues were homogenized in ice-cold phosphate-buffered saline (PBS) to prepare tissue homogenates. The levels of malondialdehyde (MDA, E- BC-K025), a marker of lipid peroxidation, were measured using the thiobarbituric acid reactive substances (TBARS) assay. Superoxide dismutase (SOD, E-BC-K022), and catalase (CAT, E-BC-K031) activities and reduced glutathione (GSH, E- BC- K051) level were determined using respective spectrophotometric assay kits from Elabscience (USA). The total protein concentration in the homogenate was determined using a total protein assay kit (Bicinchonic acid method, E-BC-K075).

5.6 Histopathological Evaluation:

Small portions of kidney tissues were fixed in 10% formalin, processed, and embedded in paraffin blocks. Sections of 4-5 µm thickness were cut using a microtome and mounted on glass slides. The sections were stained with hematoxylin and eosin (H&E) for histopathological evaluation. The slides were examined under a light microscope (Motic DMWB-1 Professional B3 Series, China) by a blinded pathologist to assess morphological changes.

5.7. Statistical Analysis

All the data were represented as mean ± standard error mean (SEM) and subjected to One-way analysis of variance (ANOVA). To compare multiple groups, Tukey's post hoc test was used. $p < 0.05$ was considered statistically significant.

Acknowledgements: Authors are grateful to IQAC, Savitribai Phule Pune University for the financial support for this study under the Assistance by SPPU for Project-based Innovative Research (ASPIRE) Scheme and to the Management of the SNJB's SSDJ College of Pharmacy, Chandwad (M.S.), India for providing necessary facility.

Author contributions: Concept – M. M., S. P., A. U.; Design – M. M., S. P., A. U., C. U.; Supervision –A. U., C. U.; Resources – M. M., A. U., C. U.; Materials – S. P., M.M.; Data Collection and/or Processing – M.M., S. P., A. U.; Analysis and/or Interpretation – M. M., A. U., C. U.; Literature Search – M. M., A. U., C. U.; Writing – M. M., S. P., A.U.; Critical Reviews – A. U., C. U.

Conflict of interest statement: "The authors declared no conflict of interest" in the manuscript.

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