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RESEARCH ARTICLE

Renoprotective effect of flavonoids in type-2 diabetes mediated-nephropathy in Wistar rats

Rohit Chettri, Prem Kumar N*

Abstract

A major problem arising from diabetes is diabetic nephropathy (DN), a fatal consequence of uncontrolled hyperglycemia characterized by progressive kidney damage caused by diabetes. Current antidiabetic therapies often exhibit side effects and fail to effectively limit the growth and advancement of diabetes. This study investigated the potential nephroprotective effects of flavonoid-rich *Emblica officinalis* fruit extract (EOFE) and hesperidin (HSD), alone and in combination, in a diabetic rodent model. After two weeks on the high-fat diet, the rats were administered a sub-diabetogenic dosage of streptozotocin to induce DN. The diabetic rats were given EOFE, low dose HSD (LDH), high dose HSD (HDH), metformin and LDH plus EOFE. Key parameters assessed included serum glucose, antioxidant levels, serum creatinine, blood urea nitrogen, proteinuria, and inflammatory markers (TNF-α, IL-6). DN biomarkers were notably elevated in untreated diabetic rats but attenuated in treatment groups, as confirmed by histopathological analysis. Notably, combined treatment with EOFE and LDH normalized nephropathic biomarkers and histopathological alterations, demonstrating effective nephroprotective activity. These findings suggest that co-administration of EOFE and LDH is a promising therapeutic strategy for managing DN, potentially addressing the limitations of current antidiabetic treatments.

Keywords: Diabetic nephropathy, *Emblica officinalis* Gaertn., Hesperidin, Antioxidant, Nephroprotective.

Introduction

Diabetes, a chronic metabolic disorder, largely entails disturbances in glucose metabolism, resulting in elevated blood sugar levels (Suganya *et al*., 2012). This is predominantly the result of insulin resistance or insufficient insulin secretion (Sheikh *et al*., 2016), which can lead to diabetic nephropathy (DN). DN is one of the serious complications of having high blood sugar for a prolonged time and not controlling it properly (Samsu *et al*., 2021). DN is linked to a higher risk of death in diabetic patients and is a progressive disease of the nephron, which is the functional unit of the kidney. It causes changes in the normal anatomy and physiology of the kidney (Elum *et al*., 2023) that lead to a drop in the glomerular filtration rate, an abnormal release of protein in the urine (albuminuria), increased peripheral fluid retention, and higher arterial tension (Samsu *et al*., 2021). Long-term high blood sugar and high blood pressure (hypertension) change the structure and function of the nephrons (Giunti *et al*., 2006). The intracellular communication channels within the nephrons are often changed in those with diabetes, which greatly helps to initiate DN, 5′ adenosine monophosphate-activated protein kinase located in the kidney, crucial for key renal functions, including ion transport & maintaining podocyte health suffering a major alteration (Hallows *et al*., 2010; Li *et al*., 2020).

Oxidative stress—caused by an unevenness between free radicals & endogenous antioxidant enzymes—damages kidney cells, proteins, &lipids. This damage accelerates kidney inflammation & fibrosis, worsening kidney function over time (Jin *et al*., 2023). Also, cytokines such as TNF-α and IL-6 cause inflammation, which affects the kidney's ability to work, initiating oxidative processes that make DN worse (Donate *et al*., 2015). Studies show promising results with the use of antioxidants sourced from natural plants in preventing oxidative damage in preclinical diabetic models (Robertson *et al*., 2023; Akpoveso *et al*., 2023).

Hesperidin (HSD), a natural bioflavonoid present abundantly in citrus fruits (Pyrzynska *et al*., 2022), is reported

Department of Pharmacology, Krupanidhi College of Pharmacy, Bengaluru, Karnataka, India.

***Corresponding Author:** Prem Kumar N, Department of Pharmacology, Krupanidhi College of Pharmacy, Bengaluru, Karnataka, India, E-Mail: premkrupanidhi@gmail.com

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to offer protection against reperfusion injury in a rat model mainly through its antioxidant properties. It is also hypothesized to attenuate renal injury mediated by the generation of free radicles in the high-fat diet (HFD) - low dose streptozotocin (STZ) animal model of diabetes. *Emblica officinalis* Gaertn. Fruit commonly named amla is an ethnic medicinal plant of India (Pandey *et al*., 2013) which has been reported for its hypolipidemic, glucose-lowering (Kim *et al*., 2005) & free radicle scavenging activity (D'Souza *et al*., 2014 & Kumar *et al*., 2009). Based on this reported information, the current investigation is being carried out to validate if the flavonoid-rich *E. officinalis* fruit extract (EOFE) and HSD, a plant phyto-constituent, can work together to protect the kidneys from DN caused by type 2 diabetes in rats.

Materials and Methods

STZ was bought from Yucca Enterprises, Mumbai, India. The flavonoid-rich EOFE was gifted by Green Chem Plot No 24D2E4, Attibele Industrial Area, Anekal Taluk, Bengaluru-562107 (Batch NO: EOE/RD/01).

Animals for In-vivo Experiment

The Institutional Animal Ethics Committee (IAEC) approved the study protocol, with the reference number KCP/IAEC/PCOL/131/AUG 2023. Fifty male Wistar rats (160–180 g) were chosen from the Animal House run by Krupanidhi College of Pharmacy, Bengaluru, India, for the study. As per the guidelines set by the Committee for Control and Supervision of Experiments on Animals (CCSEA), the animals were housed in a research facility with adequate ventilation, a cycle of 12 hours of light and darkness, an ambient temperature of 22 ± 5°C, and a humidity level of 55 ± 5%. Rats were allowed unlimited access to standard pellet food and unlimited water prior to their dietary manipulations.

Experimental Design

The rats are allocated into seven groupings, each comprising 6 rats. The normal control group (Group A) received a standard pellet diet. The remaining animals were divided into groups (Group B to G), and subjected to a dietary manipulation involving a modified HFD for two weeks, after which STZ (35 mg/kg*/i.p*) was administered to produce diabetes (Kumar *et al*., 2009; Srinivasan *et al*., 2005). Group B animals were labeled as diabetic controls. Diabetic animals across group C to G were administered orally with their designated standard drug/extract for seven weeks after STZ injection. The phytochemical and herbal essence was solubilized in 0.5% weight/volume carboxy methyl cellulose, low-dose HSD (LDH) (25 mg/kg), high-dose HSD (HDH) (50 mg/kg) (Mahmoud *et al*., 2015), flavonoid-rich methanolic EOFE (10 mg/kg) (Kumar *et al*., 2009), metformin (70 mg/kg) (Zhang *et al*., 2017). Groups C to G received a combination of flavonoid-rich methanolic EOFE and LDH, respectively, for a ten-week period.

Characterization of Diabetes Induction

The AccuCheck (Glucometer-Roche Diagnostics India Pvt. Ltd.) was used to estimate the blood glucose levels from the vital fluid that was collected by tipping the tail. Rats exhibiting a postprandial blood glucose concentration > 250 mg/dl were confirmed as successful induction of diabetes and were incorporated into the therapeutic regimen. After completion of 10 weeks of treatment protocol (termination of treatment), blood was withdrawn and serum was isolated & refrigerated for biochemical analysis using ready-made kits (Erba Diagnostics, India).

Characterization of Diabetic Nephropathy

To assess renal dysfunction and the extent of DN, blood urea nitrogen, creatinine, and proteinuria (24 hours of urine protein) were estimated as outlined earlier (Kaikini *et al*., 2020).

Inflammatory Mediators and Cytokines

A blood sample was collected & sent to Biocorp Scientific Bengaluru, India, for analysis of inflammatory cytokines, specifically TNF-α and IL-6, which are crucial in altering the normal nephron structure and have key functions in the etiopathology of diabetic kidney injury (Donate *et al*., 2015).

Renal Antioxidant Status

Superoxide dismutase

A kidney homogenate was produced by homogenizing 100 mg of renal tissue in 10 mL of 100 mM KH_2PO_4 buffer with 1 mM EDTA, with a pH of 7.2. The protein supernatant was obtained by centrifuging the preparation at 1200 x g for 30 minutes at 4°C. About 1-mL of Na_2CO_3, 0.4 mL of nitro blue tetrazolium (NBT), and 0.2 mL of EDTA were added to 100 µL of the preparation. Prior to initiating the operation, a spectrophotometric investigation was undertaken at 560 nm. The procedure started off by introducing 0.4 mL hydroxylamine HCl at a level of 1 mM. The mixture was exposed to residence at an ambient temperature of 25°C over a period of 5 minutes, after which the corresponding decrease of NBT was detected at 560 nm. A control solution was produced in the absence of preparation. The enzyme activity of superoxide dismutase was measured in units/mg of protein. A single unit of effectiveness is characterized by the amount of proteins present in 100 µL of a 10% tissue homogenate that is necessary to prevent the decline of 24 mM NBT to half (Kono *et al*., 1978).

Catalase

Spectrophotometric analysis was conducted at 240 nm, incorporating 2 mL of phosphate buffer at pH 7 with 100 µL of renal tissue homogenate. About 1-mL of H_2O_2 was added to the above-mentioned mix and allowed to rest at normal temperature for a few minutes. Absorbance was made against a buffer of phosphate as blank. 1 IU of catalase

was added to initiate the breakdown of 1 mM H_2O_2/min at body temperature and was quantified as units/mg of protein (Stevens *et al*., 2000).

Lipid peroxidation

Add 0.5 mL of renal tissue homogenate and 3 mL of phosphoric acid to 1-mL of 0.6% thiobarbituric acid. The solution was then gently heated for 40 minutes. After cooling, 4 mL of n-butanol was added to the solution, and the mixture was centrifuged at 20,000 rpm for 20 minutes. Spectrophotometric analysis was conducted at 532 nm after the organic layers were separated (Devasagayam *et al*., 2003).

Histopathology of kidney

At the terminal stage of the study, all rats were methodically sacrificed by CO_2 overdose. The kidneys from each group were then weighed, preserved within a 10% phosphate-buffered formaldehyde solution, and dried using gradient alcohol soaked in paraffin blocks. Tissues fixed in paraffin were cut into 4 mm slices and prepared with periodic acid-Schiff reagents for micro-assessment of renal histology (Wang *et al*., 2011).

Statistical Analysis

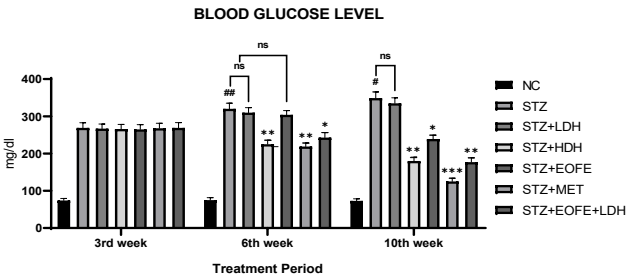
The comparisons were conducted using Dunnett's test and One-way analysis of variance (ANOVA). GraphPad Prism 10.0.2 was utilized to analyze the data, which were displayed as mean ± standard error of the mean (SEM). Less than 0.05 was the threshold for statistical significance.

Results

The early phase of the study was conducted on normal animals to ascertain the non-hypoglycemic efficacy of flavonoid-rich EOFE and HSD (Data not shown). After preliminary observations failed to show any discernible impacts on glucose levels, the above-described investigation was carried out. Several biochemical parameters were compared between normal, positive control, and treated groups (Group A-G), as mentioned below.

Effect on Blood Glucose Levels

Blood glucose levels were measured at 3rd, 6th, and 10th weeks across various treatment groups. Group B, in comparison to group A, displayed a significant increase in blood glucose levels, rising from 269 mg/dl in the 3rd week to 349 mg/dl by 10th week. Treatment with LDH did not produce significant changes throughout the treatment period. HDH, in contrast to diabetic control, exhibited a substantial decrease in glucose levels during the 6th and 10th weeks ($p < 0.01$). EOFE showed a significant effect only in the 10th week ($p < 0.05$), while the metformin-treated groups exhibited a highly significant reduction in blood glucose levels by the 10th week ($p < 0.001$). Moreover, EOFE + LDH also demonstrated observable glycemic control ($p < 0.01$), as shown in Figure 1.



Comparisons with the normal group are indicated by #$p < 0.001$ and ##$p < 0.01$, while comparisons with the STZ control group are indicated by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. *p-values* are reported as (mean ± SEM), n = 6; where ns=non-significant.

Figure 1: Effect of therapy on blood glucose level

Effect on Serum Creatinine and BUN level

After 9 weeks of therapeutic intervention, diabetic rats (Group B) showed elevated levels of creatinine (Figure 2A) and BUN (Figure 2B), contrasted with normal control (Group A). Diabetic rats administered with HDH offered a notable fall during the 6th week and a much better impact during the 9th week ($p < 0.01$), whereas EOFE had a significant effect only during the 9th week ($p < 0.05$). The LDH+EOFE treated group also showed significant improvements during the end of the trial, while metformin produced better results compared to their individual treatments ($p < 0.001$).

Effect on Proteinuria

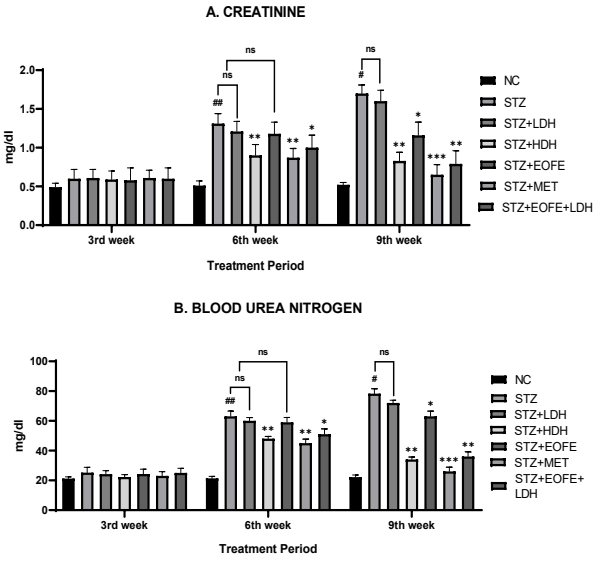
Urinary excretion of proteins in diabetic rats was higher. In diabetic rats, EOFE only showed its effect during the 9th-week interval; more pronounced results were seen with HDH (Group D) ($p < 0.01$). By the end of the 9th week, the combination therapy of EOFE and LDH (Group G) significantly delayed the onset of proteinuria compared to untreated diabetic rats, as depicted in Figure 3.

Effect on Inflammatory Markers

Compared to the normal control group, the diabetic control group exhibited significantly elevated levels of pro-inflammatory markers IL-6 and TNF-α, which indicates that the inflammation is related to diabetes. Treatment with LDH (25 mg/kg) + EOFE (10 mg/kg) reduced IL-6 and TNF-α levels, indicating their ability to reduce diabetes-induced inflammation. HDH (50 mg/kg), combination therapy, and metformin (70 mg/kg) had a strong anti-inflammatory effect, as seen in Figure 4.

Renal Antioxidant Status

There was a substantial spike in thiobarbituric acid-reactive substance (TBARS) in rats with diabetes, indicating enhanced lipid oxidation and oxidative stress. Simultaneously, there was a notable decrease in antioxidant enzyme levels in contrast to normal control. This indicates a compromised



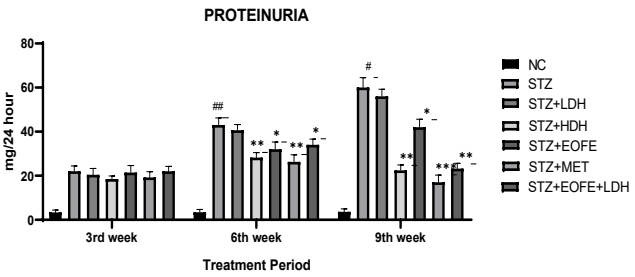
The *p-values* are presented as (mean ± SEM), n = 6. Comparisons with the normal group are denoted by #p < 0.001 and ##p < 0.01, while comparisons with the STZ control group are represented by *p < 0.05, **p < 0.01, and ***p < 0.001, where ns = non-significant.

Figure 2A & B: Effect of treatment on creatinine and blood urea nitrogen levels, respectively

level of antioxidant defense. The groups treated with EOFE + LDH and metformin (70 mg/kg) significantly reduced TBARS levels, indicating relief from oxidative strain. Diabetic rat's antioxidant capacity was further enhanced by these therapies, which also raised superoxide dismutase and catalase levels. The combination therapy of LDH and EOFE had a stronger therapeutic effect than either HSD or EOFE alone. It lowered TBARS levels more significantly and increased superoxide dismutase, catalase, and metformin activity more significantly, as depicted in Figure 5A & B.

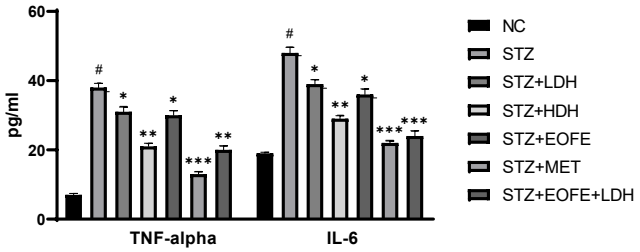
Histopathological Analysis

In comparison to normal control, diabetic control exhibited significant structural changes in the kidneys, including tubular hyperplasia, necrosis with congestion, accumulation of inflammatory cells, namely polymorphonuclear leukocytes (PMN), glomerular fibrosis, and tubular necrosis with inflammation. To counteract these diabetes-induced degenerative changes in the glomeruli, treatments with LDH, HDH, EOFE, and combinations were administered over a period of seven weeks. Among these, the combined treatment of EOFE and LDH demonstrated remarkable protection against degenerative structural defects in the kidneys, proving to be more effective compared with standalone treatment. The nephron-protective effects of this combined therapy were evident from the restoration of kidney structure in diabetic rats, reversing the diabetes-induced alterations in renal architecture as depicted in Figures 6 A-D.



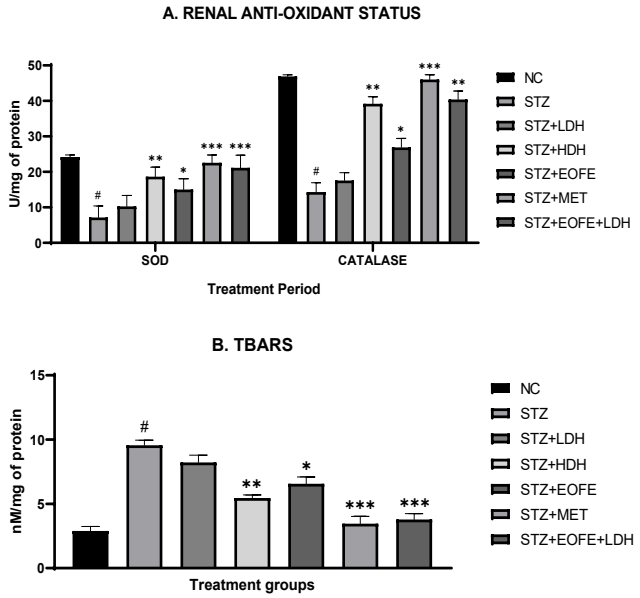
p-values are presented as (mean ± SEM), n = 6. Comparisons with the normal group are denoted by #p < 0.001 and ##p < 0.01, whereas comparisons with the STZ control group are denoted by *p < 0.05, **p < 0.01, and ***p < 0.001), where ns=non-significant.

Figure 3: Effect of treatment on proteinuria estimation



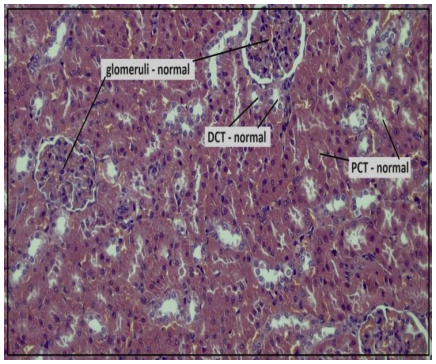
p-values are denoted as (mean ± SEM), n = 6. #p <0.001 and ##p <0.01 in comparison to the normal group; *p <0.5, **p <0.01, ***p <0.001 in comparison to the STZ control group, where ns=non-significant.

Figure 4: Effect of treatment on Inflammatory markers

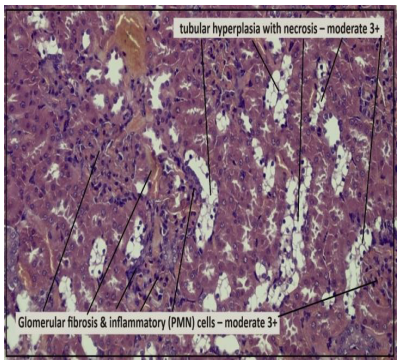


p-values are reported as (mean ± SEM), n = 6. *p <0.5, **p <0.01, and ***p <0.001 signify significance relative to the STZ control group, while #p <0.001 and ##p <0.01 denote significance relative to the normal group, where ns=non-significant.

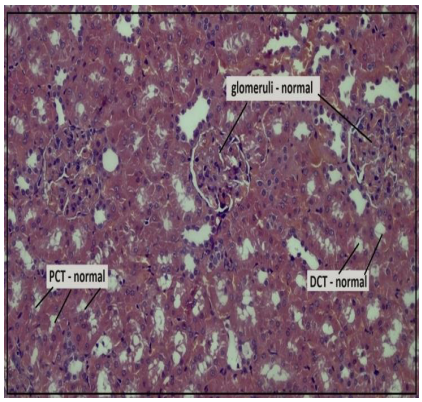
Figure 5A & B: Effect of therapeutic intervention on the levels of antioxidant status and TBARS, respectively



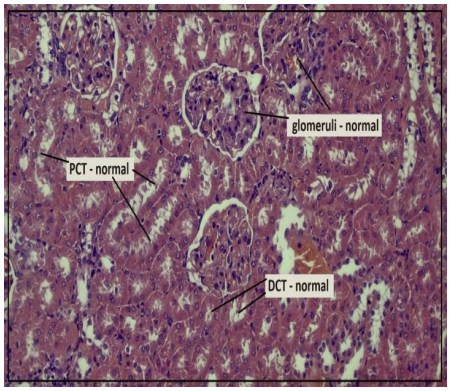
(A) Normal control – Rat kidney: showing normal glomeruli, vessels and tubules-NAD^+ at (X100)



(B) Diabetic control – Rat kidney: showing: tubular hyperplasia with congestion and accumulation of inflammatory (PMN) cells – moderate 3+ at (X100)



(C) Metformin treated – Rat kidney: showing: Renal architecture within limit at (X100)



(D) EOFE+LDH treated– Rat kidney: showing: Normal renal architecture with mild accumulation of inflammatory cells at (X100)

Figure 6: Histopathological analysis of rat kidney

Discussion

T2DM is indeed a growing global threat, and with increasing cases, diabetes-related complications are also on the rise. Chronic hyperglycemia and associated metabolic disturbances accelerate kidney dysfunction, such as DN. Despite the advancements in treatment and prediction (Raja *et al*., 2024; Ramyaveni *et al*., 2024), there is still no therapy that completely prevents renal damage in diabetic patients. Current antidiabetic and anti-hypertensive approaches only help in reducing the risk but cannot entirely halt kidney deterioration.

In the present investigation, we sought to examine the extent of renal dysfunction and the therapeutic impact of HSD and flavonoid-rich EOFE, administered either alone or in combination, in T2-diabetic rats. The study assessed several biochemical markers, including blood glucose, blood urea nitrogen, creatinine, IL-6, TNF-α & proteinuria, alongside oxidative stress markers in renal tissues. Blood urea nitrogen and creatinine are metabolized waste products eliminated via kidneys. Blood urea nitrogen serves as a marker for protein breakdown, while creatinine reflects muscle breakdown. Diabetic rat kidney function was evaluated using both creatinine and blood urea nitrogen levels. These markers are well-established indicators with elevated levels directly correlating with the degree of damage and function, signifying potential kidney impairment or damage (Al-Hazmi *et al*., 2020). Microalbuminuria serves as a pre-marker of kidney deterioration in diabetic patients, often preceding severe renal impairment. Abnormal urinary protein secretion is associated with inflammation in tubular cells and a slower progression of interstitial fibrosis and tubular atrophy (Wolf *et al*., 2007). Numerous investigations on antioxidants have demonstrated the restoration of renal function in diabetes-mimicking models (Gupta *et al*., 2020; Sun *et al*., 2021). Giving the well-known antioxidant EOFE resulted in a marked drop in blood urea nitrogen and creatinine (EL-Gawish *et al*., 2023); HSD also caused a drop in serum levels of urea nitrogen and creatinine, which made diabetic rats' blood sugar levels rise (Manasa *et al*., 2022). The combination therapy of EOFE and LDH resulted in a significant reduction in creatinine and

blood urea nitrogen levels (Figure 2A & B), along with a decrease in protein secretion in the urine (Figure 3).

Chronic oxidative damage can lead to the development of vascular disorders. High levels of reactive oxygen species (ROS) over time can result in endothelial damage, inflammation, and impaired blood vessel function, which are critical contributors to the emergence of problems related to vasculature, especially in conditions like diabetes (Yang *et al*., 2024). The antioxidant defense system, which is made up of two enzymes (superoxide dismutase and catalase), controls how cells handle superoxide radicals. Reactive hydroxyl radicals arise when the antioxidant defense systems fail to suppress superoxide radicals. Excessive amounts of these free radicals can harm cells by attaching to their proteins and nucleic acids, causing cell damage (Obrosova *et al*., 2003). In this study, the activities of endogenous superoxide dismutase and catalase were assessed in kidney tissues to describe changes in antioxidant status. Renal tissues in the diabetic group showed decreased superoxide dismutase and catalase levels in contrast to normal control. Research has revealed that lipid oxidation is markedly elevated in both diabetic subjects and lab-created diabetic rats. Increased oxidative stress unmistakably contributes to the progression and outcomes of diabetes (Ito *et al*., 2019), amplifying oxidative damage in streptozotocin-induced hyperglycemia. Rais *et al*. (2024) and Gilani *et al*. (2021) have previously reported similar findings. They found significantly higher TBARS levels in diabetic rats, implying that increased lipid peroxidation may be a significant factor for the emergence of diabetes and its complications. These harmful changes in the glomeruli were slowed down by treatment with flavonoid-rich EOFE and LDH. Ansari *et al*. (2014) and Jayaraman *et al*. (2018) hypothesized that the antioxidant capabilities of EOFE and HSD influenced their nephroprotectiveness. A substantial decrease in lipid peroxidation and a decrease in TBARS were observed as a result of combination therapy (Basavarajappa *et al*., 2020; Kumar *et al*., 2009; Ashafaq *et al*., 2014). By lowering renal TBARS, therapy with EOFE or HSD modestly decreases strain generated by reactive oxygen molecules in diabetes-mediated damage to the renal cells. The amounts of superoxide dismutase-catalase were likewise raised simultaneously by these treatments (Figure 5A & B). Thus, it appears that the antioxidative qualities of HSD, EOFE, and the two of them combined together can safeguard the nephrons. In order to offer protection against the harmful development of DN, EOFE and HSD are combined with the aim of decreasing ROS. The current study reports that the nephroprotective effect of flavonoid-rich EOFE combined with LDH was found to be effective in suppressing DN in rats compared to its standalone treatment. More glucose absorption leads to more reactive oxygen molecules being made, which damages the kidneys more (Samsu *et al*., 2021). Elevated oxidative damage stimulates the synthesis of extracellular matrix constituents (Mason *et al*., 2003), which in turn leads to the expansion of mesangial cells, thereby altering the filtration process. Histopathological investigations demonstrated a proliferation in glomerular mesangium in uncontrolled diabetic rodents compared to healthy rodents. These harmful changes in the glomeruli were slowed down by treatment with flavonoid-rich EOFE and LDH. It is thought that the antioxidant capabilities of EOFE and HSD influence their nephroprotectiveness (Ansari *et al*., 2014; Jayaraman *et al*., 2018).

Chronically high blood sugar levels are a major cause of DN. This is because they cause more free radicals to be released because glucose is broken down more, the sorbitol pathway is stimulated, glucose is oxidized, and glycosylation happens without enzymes. Oxidation of glucose produces toxic keto-aldehydes and superoxide radicals (Obrosova *et al*., 2003). In the current study, normalizing blood glucose levels with the proposed combination was not possible even at the end of the 10^{th} week (<0.01), which necessitated the need for prolonged treatment (Figure 1), thus the protective effect for the kidneys in nephropathic rats can be attributed to the synergism between antioxidant status and blood glucose level.

Cytokines linked to inflammation are important in the range of processes related to DN, from the onset of diabetes to advanced kidney failure (Donate *et al*., 2015). Diabetic rats that were not administered any treatment exhibited elevated levels of TNF-α and IL-6. These levels are linked to kidney damage and inflammation between cells. This finding holds true for people with diabetes who have developed DN (Araújo *et al*., 2020). As demonstrated by histological investigation, the suggested combination therapy, mainly by virtue of their antioxidant activity, was successful in reducing the IL-6 levels linked to extracellular matrix synthesis and glomerular basement membrane thickness. Similar findings were found on TNF-α's role in tubular injury, which led to lower levels of albumin in the urine and glomerular and tubular damage in DN rats compared to those that were treated with a drug alone (Figure 4). The proposed combination is a promising therapeutic option for a combinational regimen, as it mitigates the risk of hypoglycemia that is associated with the ongoing use of conventional oral hypoglycemic agents and offers protection against diabetic complications that are exacerbated by oxidative stress.

Conclusion

In conclusion, the administration of EOFE + LDH has shown promising nephroprotective effects in DN. These effects are likely mediated through the normalization of key nephropathic markers and the significant reduction of oxidative strain. Oxidative stress plays a critical role in diabetes-related complications, and its mitigation is

essential in preventing the progression of the disease. Moreover, lifestyle modifications that include physical exercise and supplementation of dietary flavonoids may allow for the withdrawal of metformin.

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