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RESEARCH ARTICLE

Polyphenolic compounds as novel reno-modulatory agents in the management of diabetic nephropathy in Wistar rats

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Abstract

Diabetic nephropathy (DN), a prevalent diabetes-related complication that leads to end-stage kidney failure due to functional changes in the renal system. Contemporary antidiabetic interventions effectively fail to prevent or slow down the course of diabetic kidney damage. The current study investigates the impact of *Coccinia indica* ethanolic leaf extract (CILE) both individually and combined with hesperidin, a bioflavonoid, to mitigate the advancing renal damage induced by non-insulin-dependent diabetes mellitus in a rodent model. DN was triggered in experimental rats by administering a high-fat diet and, thereafter, by a single injection of streptozotocin (35 mg/kg, intraperitoneally). The experimental groups received CILE, hesperidin (high and low dose), *C. indica* in conjugation with low-dose hesperidin and metformin alone during the treatment period. The study evaluated several parameters, including serum glucose, antioxidant levels, blood urea nitrogen, urine protein levels, creatinine, and inflammatory mediators (interleukin-6 and tumor necrosis factor-alpha). These parameters were also studied in negative control animals to understand and compare the therapeutic potential of polyphenolic compounds. The histomorphological study coincides with the elevated biomarkers observed in the non-intervention groups. The treatment with different predetermined therapies normalized all biomarkers toward normal levels, including histopathological changes. Therefore, administration of hesperidin and CILE showed significant benefits in delaying the progression of kidney damage and presents promising potential therapy for managing DN.

Keywords: Diabetic nephropathy, *Coccinia indica*, Bioflavonoid, Streptozotocin, Metformin, Inflammatory markers.

Introduction

Diabetes is a chronic, complex, and still incurable public health issue that impacts individuals worldwide (Su *et al.,* 2023). With millions of cases worldwide, diagnosing the likelihood of diabetes type 2 development is vital for early action and management (Raja & Nagarajan, 2024). Contemporary lifestyles and external influences are the primary causes of type 2 diabetes mellitus (T2DM). Perpetual hyperglycemic is a defining characteristic of diabetes, a protracted metabolic pathology that can persist over extended temporal durations. Elevated blood sugar produces the symptoms of an increase in urination frequency, persistent thirst, and insatiable hunger (Kumar *et al*., 2020). The Langerhans islets, which produce insulin, become less active, and thus, glucose in the blood rises (Khin *et al*., 2023). Diabetes complications can escalate due to uncontrolled blood sugar levels, resulting in 20 to 50% of patients developing diabetic nephropathy (DN), an advanced kidney disease associated with arterial hypertension, cardiovascular-related morbidity, and mortality (Selby & Taal, 2020). Patients suffering from DN often suffer from kidney dysfunction, including thick glomerular membranes, glomerulosclerosis, loss of podocyte adhesion, cell growth, fibrosis, and larger glomeruli (Arora *et al*., 2010). End-stage kidney disease is generally marked by the unusual occurrence of microalbuminuria and an increase in the glomerular filtration rates, which are nearly double the normal range. The progression of DN is also triggered by growth factors such as PDGF-β, TGF-β1 and connective tissue growth factors that cause alteration in the glomeruli (Schena & Gesualdo, 2005). Some miRNAs have been identified as potential biomarkers for DN diagnosis, prognosis, and therapeutic targets, such as miRNA 181a. Impaired or abnormal functioning of some miRNA leads to

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insulin resistance and obesity (Hussain *et al*., 2024). Oxidative stress contributes to disease progression, including DN, by disrupting homeostasis and causing cell damage. In decompensated T2DM, impaired renal function worsens oxidative stress, leading to chronic inflammation and nephropathy. Monitoring oxidative stress and inflammation can help manage complications and improve patient health (Goycheva *et al*., 2023). Hesperidin effectively treats diabetic neuropathy and nephropathy by normalizing blood glucose and lipid levels. Hesperidin offers antioxidant, anti-inflammatory, and neuroprotective benefits, making it promising for diabetes treatment (Mirzaei *et al*., 2023).

"Perennial climber," widely known as *Coccinia indica* has garnered considerable prominence in recent decades owing to its high therapeutic benefits and was extensively used in traditional medicine (Chinni *et al*., 2021). *C. indica* is rich in a diverse array of phytoconstituents. These include flavonoids, phenols, fixed oils, glycosides, alkaloids, tannins, saponins, amino acids, carbohydrates, fats, and proteins. Each of these bioactive compounds contributes to the plant's medicinal properties, making it valuable for various therapeutic applications (Selvaraj, 2024). Due to its effectiveness in regulating blood sugar levels, *C. indica* is now regarded as a traditional alternative to insulin (Basavarajappa *et al*., 2020). Theoretically, its free radical-scavenging abilities ameliorates kidney injury and regulate lipid oxidation, potentially reducing oxidative assault in diabetes caused by streptozotocin (Venkateswaran & Pari, 2002). It has been extensively used for various conditions due to its anti-inflammatory, antinociceptive, neuroprotective, analgesic, hepatoprotective, hypoglycemic, and antilipidemic properties (Niazi *et al*., 2009; Padma & Vinoth, 2022). *C. indica* is also utilized in treating tumors (Kumari *et al*., 2016). The therapeutic impact of *C. indica,* when co-administered with a low dose of pioglitazone-managed DN, was demonstrated earlier (Basavarajappa *et al*., 2020). Hesperidin has been shown to exert a protective effect in the prevention of DN in streptozotocin (STZ) induced diabetic rats (Manasa & Suhasin, 2022).

Since the combination of *C. indica* and hesperidin has not been carried out, this research uniquely highlights the potential impact of *C. indica* ethanolic extract (CILE), hesperidin (low and high dose), metformin, and a combination of CILE and low dose hesperidin on diabetic kidney disease. The aim of this study was to investigate whether CILE, either alone or in combination with low-dose hesperidin, can delay the progression of kidney damage induced by type II diabetes in a rat model.

Materials and Methods

Chemicals

Streptozotocin (STZ) was purchased from Yucca Enterprises, Mumbai.

Preparation of Flavonoid Rich Ethanolic Leaf Extract of C. indica

Leaves of *C. indica* were allowed to dry before extracting them using ethanol. The material was heated in a closed chamber for 5 to 6 hours throughout the extraction process, where *C. indica* was re-pumped into the herb bed. Two iterations of this process were carried out. At a low temperature and with decreased pressure, the resultant extracts were then mixed and concentrated. The condensed extract was moved to an evaporation unit, where it underwent drying and was subsequently collected as a fine powder. The material was processed into a fine powder using a multimill and then thoroughly blended to ensure a consistent, homogenous mixture (Basavarajappa *et al*., 2020).

Test Animals

The present study was approved by the Institutional Animal Ethics Committee (Krupanidhi College of Pharmacy, Bengaluru, Karnataka, India) bearing Approval No. KCP/IAEC/PCOL/133/AUG-2023. The study involves the use of male Wistar rats weighing between 200 to 250 g. The animals were acclimatized to standard laboratory conditions. Briefly, the experimental rodents were maintained in a controlled environment with temperatures ranging from 25 to 29°C, relative humidity (55–60%), and a regulated 12-hour light/dark photoperiod. The animals were offered unlimited provision of water and maintained a standard diet prior to their dietary changes. Prior to commencing to experimental investigation, the animals underwent a preliminary procedure to ascertain the effect of the treatment protocol on glycemic control (Basavarajappa *et al*., 2020).

Experimental Design

The study entails random assignment of the experimental animals into seven distinct groups, with each group consisting of six animals. Animals delineated into the normal control as group I were given a standard pellet diet. For two weeks, animals in groups II to VII were given a fat-rich diet and then one-time administration of STZ (35 mg/kg) through the intraperitoneal route to induce diabetes. Post-diabetes induction, groups III to VII received their respective standard drugs or extracts orally for eight weeks. To dissolve the phytochemicals and plant extract, carboxymethyl cellulose (0.5% w/v solution) was utilized. Group II represented the diabetic control group; animals in group III were given CILE (200 mg/kg) (Basavarajappa *et al*., 2020). Groups IV and V were administered with lower and higher (25 & 50 mg/kg) dosages of hesperidin, respectively (Visnagri *et al*., 2014). Group VI was treated with a combination of hesperidin (low dose) and CILE, while group VII was treated with 70 mg/kg metformin (Zhang *et al*., 2017). Blood samples were collected on a weekly basis throughout eight weeks.

Development of Diabetes (type II) in Rats

Experimental animals were given access to a diet rich in fat for a duration of two weeks. After this period, the rodents

were fasted overnight, followed by an intraperitoneal injection of STZ (35 mg/kg/i.p). Blood samples were retrieved from the rodent's tail vein and analyzed for blood glucose concentrations using the Accu-Chek glucometer (Roche Diabetes Care India Pvt. Ltd.). Rats with non-fasted sugar levels surpassing 250 mg/dL were classified as diabetic. Furthermore, the investigation included animals exhibiting glucose levels higher than 250 mg/dL (Basavarajappa *et al*., 2020).

Assessment of Diabetes-related Renal Impairment

The extent of nephropathy caused by diabetes was assessed through biochemical measurement of blood creatinine levels, protein in urine, and blood urea nitrogen (BUN). Serum creatinine was analyzed using the alkaline picrate method (Bonsnes & Taussky, 1945). For the assessment of serum urea, the end-point method (Fawcett & Scott, 1960) was utilized, facilitated by a semi-auto-analyzer and urea reagent. These methodologies provided precise measurements of key biochemical parameters essential for evaluating renal function.

Assessment of Inflammatory Mediators

The assessment of inflammatory mediators was performed as described by Donate-Correa *et al*., 2015. Briefly, after completion of therapeutic treatment, diabetic rats were anesthetized, and blood samples were collected via cardiac puncture. The serum was separated by allowing blood to clot, followed by centrifugation, and the samples were stored at –20°C until use. Tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) levels from each sample were analyzed by sampling blood samples at Koushik Laboratories, Bengaluru, India.

Assessment of Antioxidant Status

The antioxidant factors were evaluated by adhering to a well-established protocol.

Superoxide Dismutase Estimation

The estimation of superoxide dismutase (SOD) relies on detecting oxygen through auto-oxidation of hydroxylamine hydrochloride, resulting in the generation of nitrite, which is then quantified colorimetrically at 560 nm. The SOD value is determined by measuring the degree of inhibition in nitroblue tetrazolium (NBT) reduction. NBT undergoes reduction via hydroxylamine auto-oxidation, leading to nitrite formation, which is detected colorimetrically, with EDTA included in the process. In this assay, a homogenized kidney tissue sample (100 µL) was added to a reaction vessel, followed by 0.2 M phosphate buffer, 1-mL sodium carbonate mixture 4 mL NBT, and 0.2 mL EDTA for baseline measurement using UV spectrophotometer at 560 nm. The commencement of the reaction was triggered by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. The reduction of NBT was observed at 560 nm after a 5 minutes culture period at 25^{0}C (Gao *et al*., 2023; Maroof *et al*., 2023).

Catalase Estimation

For estimation of catalase, 100 µL of kidney tissue extract prepared with 0.15 M potassium chloride solution was mixed with 1.9 mL of 0.25M buffer solution containing phosphate at pH 7. Optical density measurements were conducted at a wavelength of 240 nm. Following that, the resulting solution contained 1-mL of H_2O_2 and was kept for one minute under incubation. Absorbance was measured again using phosphate buffer as the reference sample (Stevens *et al*., 2000).

Lipid Peroxidation Estimation

The estimation of lipid peroxidation was carried out as described by Sadžak *et al*., (2021) and Angelova *et al*., (2021) with minor modifications. The quantification of lipid peroxidation involved evaluating the interaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). Standard protocol was followed to estimate thiobarbituric acid reactive substances (TBARS) in blood components. A suspension was prepared by mixing 0.5 mL of kidney tissue, 1-mL of 0.6% TBA, and 3 mL of phosphoric acid and incubated at 45°C. The resultant solution was then placed in a warm water bath and incubated for 45 minutes later, n-butanol (4 mL) was added after cooling. For 20 minutes, the fluid was subjected to centrifugation at 20,000 rpm. After being moved to a fresh test tube, the organic layer was collected to check absorbance using UV spectrometer at 532 nm.

Tissue-based Pathological Assessment

The kidneys were removed and stored in 10% formalin when the animals were sacrificed at the end of the study. After being dehydrated with ethanol, the tissues were embedded at a 5 µm thickness in paraffin. Haematoxylin and eosin staining was used for the analysis of cellular structure. Additionally, Masson's trichrome staining was utilized to detect possible collagen deposition in the tissues, and periodic acid-Schiff (PAS) reagent was used to assess modifications to the basement membrane, such as thickening and buildup of glycogen. With the observers masked to the treatment and control conditions, optical microscopy facilitated the assessment of interstitial inflammatory cell hyperplasia and morphological abnormalities (Wang *et al*., 2011).

Statistical Analysis

Following the one-way ANOVA, Dunnett's test was employed for comparison. The GraphPad Prism 10.0.2 software was used to analyze the results. Data representation was accomplished using mean ± standard error of the mean, with statistical significance set at a *p-value* of < 0.05.

Results

Impact of Treatment Intervention on Blood Glucose Level

The onset of STZ-induced hyperglycemia resulted in a substantial rise in the concentration of serum glucose,

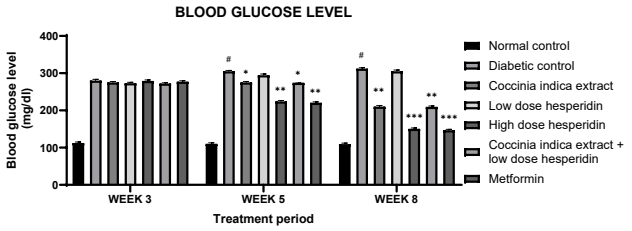
increasing more than two to threefold when compared to a normal control group. However, implementing the designed therapeutic interventions substantially mitigated this increase as shown in Figure 1. Animals receiving low-dose hesperidin produced an insignificant reduction of blood glucose concentration on 5th week, but a reduction of p <0.001 was observed with high-dose hesperidin and metformin on 8th week. However, the diabetic animals treated with CILE alone and in combination with low-dose hesperidin exhibited a significant reduction only on the 8th week (p <0.01) when compared to the 5th week of therapy. The reduction in blood glucose level with the proposed combination was showing reduction only during the 8th week upon comparison to other therapies.

Impact of Treatment Strategies on BUN and Creatinine Concentration

Elevation of BUN and concentration of creatinine was detected in disease control in correlation to negative control. Diabetic animals subjected to metformin, high and low doses of hesperidin, and CILE for a period of 5 weeks displayed a slight reduction in BUN as well as creatinine concentration in the blood, whereas no significant results were observed during the 3rd week. Reduction in the BUN and creatinine concentration was found to be more effective during the 8th week of the therapy, as seen in Figures 2 & 3, respectively. Giving metformin alone and hesperidin (higher dose) had a greater effect on lowering BUN and creatinine levels (p <0.001). On the other hand, using CILE alone and in combination with hesperidin (lower dose) had the same effect on lowering these markers (p <0.01).

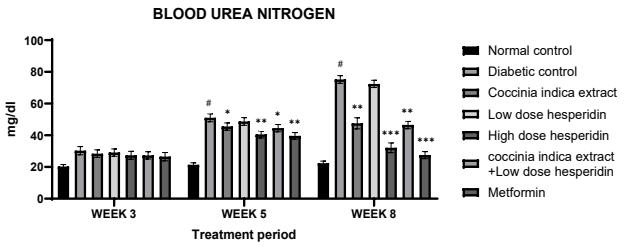
Impact of Treatment Plans on Albuminuria

Protein concentrations in the urine were quantified in the 3rd, 5th, and 8th weeks, respectively, where untreated diabetic animals showed an increase in albuminuria compared to the negative control. After 5 weeks of therapy, diabetic animals receiving high doses of hesperidin and metformin exhibited a reduction in urinary protein levels. Treatment with CILE alone, as well as in combination with hesperidin (low dose), moderately reduced urinary protein concentrations.



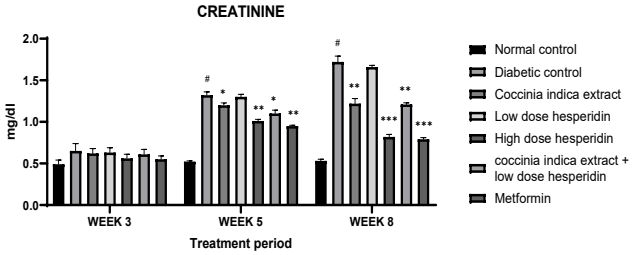
The concentration of blood glucose was measured in different weeks and was expressed in mg/dL. Findings are displayed as mean value ± standard error; n=six test rodents, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 in contrast to disease control, and $^{\#}p$ <0.001 in correlation to negative control.

Figure 1: Effect of treatment on blood glucose level



Concentration of BUN was measured in different weeks, where findings are displayed as mean value ± standard error; n= six test rodents, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 in contrast to disease control and $^{\#}p$ <0.0001 in correlation to the negative control.

Figure 2: Effect of treatment on BUN



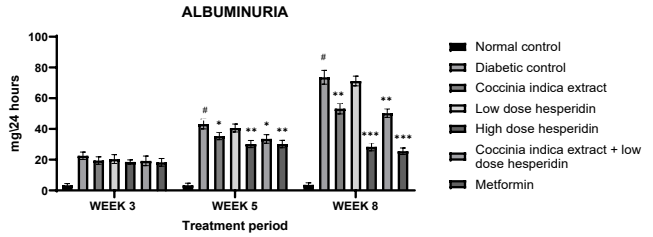
The level of creatinine was assessed in each group in various weeks, where findings are displayed as mean value ± standard error; n= six test rodents, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 in contrast to disease control, and $^{\#}p$ <0.0001 in correlation to negative control.

Figure 3: Effect of treatment on creatinine

The 8th week of treatment showed significant results. Animals treated with metformin and high doses of hesperidin stopped the release of urinary proteins significantly (p <0.001). This was in contrast to animals treated with the CILE alone and in combination with a low dose of hesperidin (p <0.01). Animals receiving low doses of hesperidin showed no significant effect on albuminuria, was observed in Figure 4.

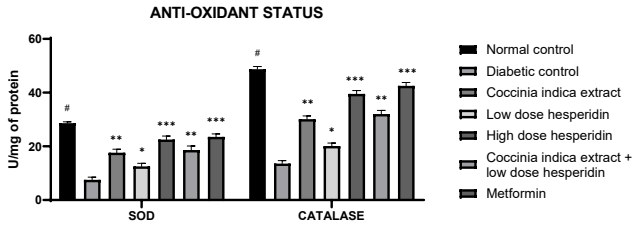
Evaluation of Pharmacological Treatments on Antioxidant Enzymes in Kidney

In correlation to negative control rats, disease rats showed considerably lower enzymatic activity of SOD and catalase, which were followed by a considerable augmentation in MDA levels in renal tissue as a result of oxidation of lipids. Hesperidin, CILE, and metformin administration to the diabetic rats resulted in a decrease in TBARS concentration and a concurrent increase in antioxidant activity in the renal tissues. This ultimately leads to a decrease in the oxidative damage. Interestingly, CILE and, when combined with low-dose hesperidin, had a significant effect (p <0.01). However, high-dose hesperidin and metformin had a better therapeutic effect (p <0.001), showing a more noticeable



The levels of albuminuria were assessed in each group in different weeks, where findings are displayed as mean value ± standard error; n = six test rodents, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 in contrast to disease control, and $^{\#}p$ <0.001 in correlation to negative control.

Figure 4: Effect of treatment on albuminuria



Estimation of SOD and catalase activity was conducted in each group. The results are displayed as mean value ± standard error; n= six test rodents, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 in in contrast to disease control and $^{\#}p$ <0.001 in correlation to negative control.

Figure 5: Effect of treatment on renal antioxidant status

decrease in TBARS and a greater rise in SOD and catalase activity as seen in Figure 5 & 6, respectively.

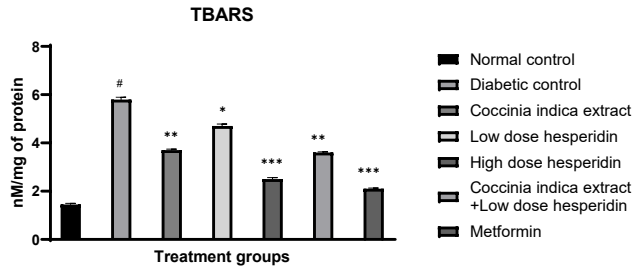
Impact of Treatment Strategies on Inflammatory Mediators

Concentrations of inflammatory markers, specifically TNF-alpha and IL-6, were quantified in all the experimental animals. Diabetic rodents showed elevated levels of these mediators, indicating increased inflammation associated with diabetes. When a low dose of hesperidin was given, the concentration of IL-6 and TNF-alpha went down slightly (p <0.05), but treatment with CILE and its combination with low dose hesperidin produced a significant decline in inflammatory markers (p <0.01). Notably, the groups that received a higher dose of hesperidin and metformin alone had a significant drop (p <0.001) in the levels of inflammatory markers as seen in Figure 7. This showed that the treatments had an anti-inflammatory effect and could reduce inflammation caused by diabetes.

Evaluation of Kidney Anatomy: Histopathology

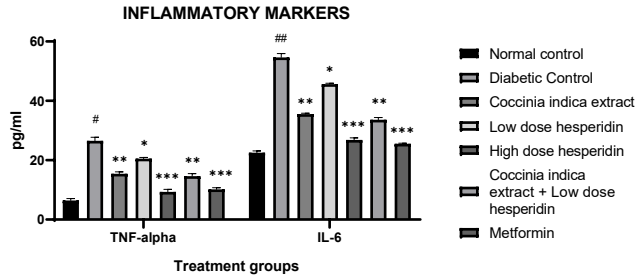
Untreated diabetic animals, upon histopathological examination revealed disruptions in the structural integrity of the kidney, including tubular enlargement, congestive necrosis, infiltration of inflammatory polymorphonuclear (PMN) cells, glomerular scarring, and tubular necrosis accompanied by inflammation. Administering hesperidin, CILE, and metformin for the duration of 8 weeks effectively prevented diabetes-related alterations in the glomeruli. Significant protection in the alteration of kidney structures was seen in animals treated with CILE. Thus, metformin and high-dose hesperidin-treated diabetic animals showed a nephroprotective effect, as seen in Figure 8 (a-d), respectively.

Histopathological findings in diabetic rats subjected to various therapeutic interventions were observed across the following groups using hematoxylin and eosin stain. DCT = distal convoluted tubules, PCT = proximal convoluted tubules.



The concentration of TBARS was assessed across different groups. The results are displayed as mean value ± standard error; n = six test rodents, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 compared to a disease control group, and $^{\#}p$ <0.0001 compared to a negative control group.

Figure 6: Effect of treatment on TBARS



The concentration of TNF-alpha & IL-6 was assessed across different groups, where findings are displayed as mean value ± standard error; n = six test rodents, $^{***}p$ <0.001, $^{**}p$ <0.01, $^{*}p$ <0.05 in contrast to disease control, and $^{\#}p$ <0.0001 in correlation to negative control.

Figure 7: Effect of treatment on inflammatory markers

Discussion

As the number of people with T2DM increased, so did the number of people with diabetes-related comorbidities. This made kidney damage and dysfunction much worse and put more stress on healthcare systems (Iskender *et al*., 2017). Diabetes is known to cause DN, which changes the structures of the kidneys and leads to terminal kidney failure

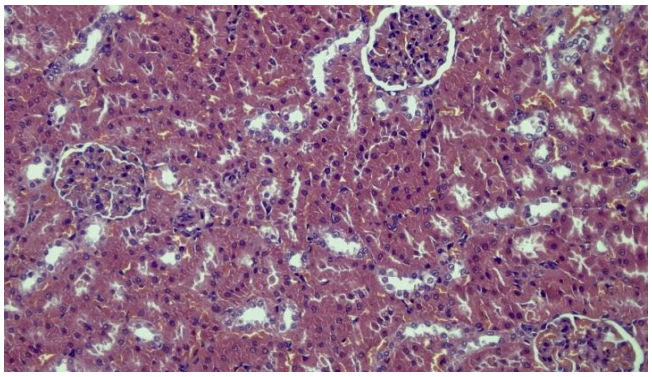


Figure 8 (a): Tissue analysis: (100X) normal control. Showing no alteration in the kidney structure. Glomeruli, urinary space and vessels within the normal range. PCT & DCT are also normal

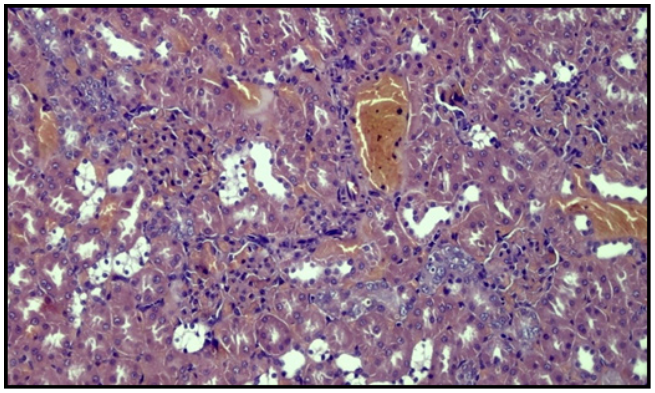


Figure 8 (b): Tissue analysis:(100X) diabetic control. Showing alteration in the kidney structure. Tubular hyperplasia with congestion, tubular necrosis and glomerular fibrosis. The medullary region showing tubular necrosis and inflammation.

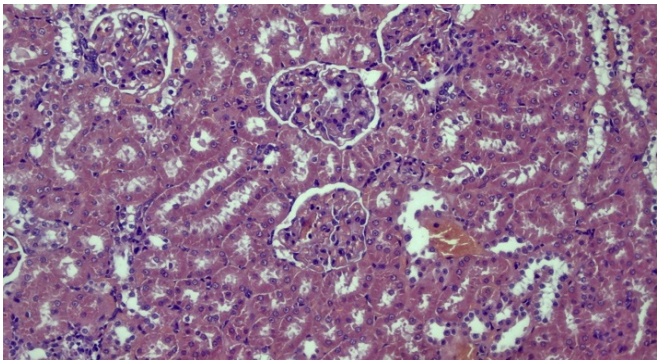


Figure 8 (c): Tissue analysis:(100X) CILE treated group. Tubular regeneration, glomeruli, PCT, DCT within normal range. The medullary region showing less inflammation and necrosis.

(Ramyaveni & Maniraj, 2024). High blood sugar levels trigger oxidative stress, which reduces antioxidant activity and raises free radical levels, leading to diabetic complications (Kishore & Singh, 2019). Oxidative stress is a fundamental contributor to the onset and progression of nephropathy. Oxidative stress makes inflammatory responses worse, which damages glomerular cells, causes fibrosis in the kidney tissue, sclerosis in the glomeruli, and degeneration

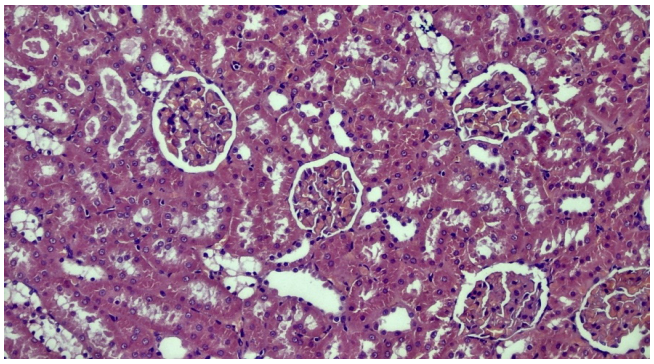


Figure 8 (d): Tissue Analysis:(100X) Metformin Treated Group. Renal architecture within normal limit, glomeruli within the normal limit PCT & DCT are normal.

of the renal tubules. All of these things make diabetes more complicated (Samsu, 2021). Activation of glucose-mediated pathways in the renal system, along with enhanced reactive oxygen species production, leads to several detrimental outcomes, including abnormal protein presence in the urine, glomerular scarring, and tubular interstitial fibrosis, all of which contribute to progressive renal damage and dysfunction (Basavarajappa *et al*., 2020). Our research looked at how CILE and hesperidin (at low and high doses) and CILE plus a lower dose of hesperidin (compared to metformin) affected blood sugar levels, kidney function markers (BUN, albuminuria, and creatinine), oxidative stress, and inflammation in diabetic rodents, specifically how TNF-alpha and IL-6 changed in kidney tissues. The experimental paradigm involves the usage of animals with diabetes (type-II) prompted by feeding a diet rich in fat and a single dose of STZ (35 mg/kg).

In our investigation, renal function was evaluated through the quantification of BUN, urea, and creatinine, metabolites that are regularly excreted *via* the kidneys. Creatinine and BUN are the by-products of muscle metabolism and protein catabolism. High blood levels of creatinine may indicate compromised kidney function. Likewise, a significant rise in BUN levels indicates renal impairment (Latini *et al*., 2016). By the 5th week, untreated diabetic animals displayed a modest increment in creatinine and BUN concentrations relative to normal animals, which progressed to a significant elevation by the 8th week. Using CILE alone or in combination with hesperidin led to a significant improvement in nephropathic markers, as shown in Figures 2 and 3, respectively.

Whether micro or macro, increasing proteinuria marks the advancement of kidney injury and scarring. Microalbuminuria denotes the finding of small quantities of albumin in the urine, a precursor to kidney dysfunction. Similar to earlier studies on DN (Basavarajappa *et al*., 2020), our study found changes in the blood and urine of diabetic animals that were not treated for eight weeks, as well as damage to the glomeruli. We measured albuminuria at the

3rd, 5th, and 8th weeks of the therapy and found that the 8th treatment intervention significantly reduced urinary protein excretion compared to the untreated controls. Higher doses of hesperidin and metformin showed much better results (p <0.001) compared to the animals that were given CILE (p <0.01), as shown in Figure 4.

Conventional treatments for DN don't work very well, but flavonoids, which are antioxidants, have shown promise in targeting multiple disease mechanisms with few side effects (Jin *et al*., 2023). According to Small *et al*. (2012), many studies using diabetes-related animal models showed that antioxidant therapy improved kidney function. Both diabetic animal models and human subjects exhibit heightened, increased concentrations of reactive oxygen species and lipid peroxidation, emphasizing the importance of oxidative stress in the mechanisms and advancement of diabetes.

In our study, we measured the amount of MDA in the kidneys and checked the activity of SOD and catalase enzymes to see how antioxidants work. MDA levels were elevated, while the activity of catalase and SOD was diminished in the kidney tissues of the diabetic group in correlation to negative control. These findings align with those previously documented by Elbe *et al.* (2015). Patel *et al*. (2015) did a full study on the redox balance in rats before and after they were given diabetes. They found that the rats in the untreated disease group had significantly higher MDA levels. Based on this, it seems that higher levels of TBARS, SOD, and catalase are very important in the development of diabetic kidney damage (Iskender *et al*., 2017). Accordingly, treatment with CILE and hesperidin produced a significant increase in catalase and SOD levels in diabetic rodents, accompanied by a decline in TBARS concentrations, upon correlating to the positive control group. This implies that the antioxidative qualities of CILE help shield the kidneys from oxidative damage.

Oxidative stress and inflammation are very important in the progression of T2DM. When they are activated by redox imbalance in different ways, they damage and scar renal tissue (Zhang *et al*., 2017). Both human and rodent model studies showcased an augmentation in the levels of IL-6 and TNF-alpha, which are essential mediators of inflammation involved in nephropathy. The untreated diabetic animals showed increased levels of these mediators, leading to more inflammation in the renal tissues compared to the negative control animals. The current research shows that administering CILE and hesperidin attenuated the upregulation of IL-6 and TNF-alpha in the kidneys, thereby reducing the inflammatory response associated with DN. Metformin treatment also yielded significant results in diseased animals.

Numerous reports suggest that therapeutic strategies aimed at boosting antioxidant capacity are critical, given the increased oxidative stress and reduced antioxidant defenses (Maciel *et al*., 2013). The activation of nuclear factor-kappa B (NF-κB) by reactive oxygen species stimulates the expression of pro-inflammatory cytokines, further exacerbating insulin resistance. IL-6 disrupts insulin signaling in hepatocytes by inhibiting the phosphorylation of insulin receptor substrate-1 (IRS-1) and activation of protein kinase B (Akt). While TNF-alpha impairs glucose uptake, increases hepatic glucose production, promotes lipolysis, and interferes with muscle insulin signalling. Addressing redox imbalance and inflammatory pathways may improve glucose control and enhance insulin sensitivity (Germoush *et al*., 2019).

Metformin is the first drug used to treat T2DM. It works well at controlling blood sugar levels, but long-term use can lead to hypoglycemia and other bad effects like kidney damage, fatigue, and stomach problems (Gökçay & Śahin, 2021; Jacob & Narendhirakannan, 2019). While metformin activates AMP-activated protein kinase (AMPK) to enhance glucose uptake and reduce hepatic glucose production, its full mechanism of action remains unclear (Chang *et al*., 2023). Additionally, CILE and hesperidin also mitigate oxidative stress by activating AMPK. When CILE and hesperidin activate AMPK, they increase the AMP/ATP ratio (Moon, 2024), phosphorylate NF-κB, facilitate its translocation, and induce the expression of antioxidant enzymes, such as heme oxygenase 1 (HO-1) (Xu *et al*., 2020). Additionally, it triggers the suppression of inflammatory cytokines (IL-6 and TNF-alpha), halting the kidney's nephrons from further degradation, as evidenced by the histopathological examination as seen in Figure 8 (a-d), respectively. In the same way, it helps glucose move around and stops the mammalian target of rapamycin (mTOR), which stops protein production and glucose production (Paul *et al*., 2022). Thus, flavonoids like hesperidin and *C. indica* improve insulin sensitivity similar to that of metformin. This is done by activating AMPK.

Additionally, AMPK also regulates the Peroxisome proliferator-activated receptors (PPARs) involved in key metabolic and inflammatory pathways. Peroxisome proliferator-activated receptor-gamma (PPARγ) activation enhances insulin sensitivity, lowers glucose and lipid levels, and attenuates inflammatory cytokines. Its capacity to mitigate oxidative stress and inflammation positions it as a promising therapeutic target in the management of metabolic diseases (Germoush *et al*., 2019).

In addition to their metabolic effects, *C. indica* (Bharti *et al*., 2018) and hesperidin (Elshazly *et al*., 2018) are said to be involved in activating PPARγ. This is linked to reduced inflammation and increased activity of antioxidant enzymes as seen in Figures 5, 6, and 7, all of which are important for slowing down the kidney damage that comes with diabetes.

The outcomes of the current research are congruent with earlier investigations by (Visnagri *et al*., 2014). This

manuscript holds significance for the scientific community as it investigates the potential of *C. indica* extract and hesperidin in addressing DN, a common complication of diabetes. Our findings indicate that treatment with CILE, hesperidin (high dose), and a combination of CILE + hesperidin (low dose), and metformin alone, significantly mitigated oxidative stress, inflammatory cytokines, and the biomarkers of DN. Parameters such as BUN, creatine, and albuminuria were observed to decrease significantly in the treated groups, suggesting a delay in the progression of DN. However, animals that were given CILE plus hesperidin (low dose) or CILE alone had similar outcomes in managing different parameters connected to DN. This signifies that hesperidin (low dose) when administered alone or in conjunction, does not produce any effect in DN.

DN is a sustained disorder and requires prolonged therapy. Recent studies in the pharmacological intervention for DN have yielded a range of pharmaceutical agents, including biguanides (metformin). Both metformin and hesperidin (high dose) have demonstrated significant effects in lowering nephropathic markers, but they are not suitable for prolonged therapy. This is mainly due to the severe adverse effects and toxic complexities related to metformin and hesperidin (high dose). Similarly, based on the obtained results, we can conclude that CILE alone significantly contributes to the progression of diabetes-mediated nephropathy. Therefore, a daily dietary intake of CILE could serve as an alternative therapy, taking the place of metformin and hesperidin, in mitigating the progression of diabetes-mediated kidney disease.

Conclusion

In conclusion, the combinatory approach utilizing CILE + hesperidin (low dose) demonstrates therapeutic equivalence to CILE monotherapy by exerting its effects through diverse mechanisms, including attenuation of oxidative stress, modulation of inflammatory pathways, and regulation of glucose homeostasis. Hence, *C. indica* leaves extract can be effectively used on its own rather than in conjunction with low-dose hesperidin. Therefore, frequent consumption of foods with these qualities may prevent and slow the progression of diabetes complexities, such as DN. Future research should explore the synergistic potential of integrating "*C. indica*" with exercise regimens in DN management, aiming to reduce or eliminate the need for metformin therapy.

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