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Protective effect of white mulberry (*Morus alba* Linn.) leaf extract against inflammatory responses and oxidative stress in obstructive nephropathy-related kidney damage

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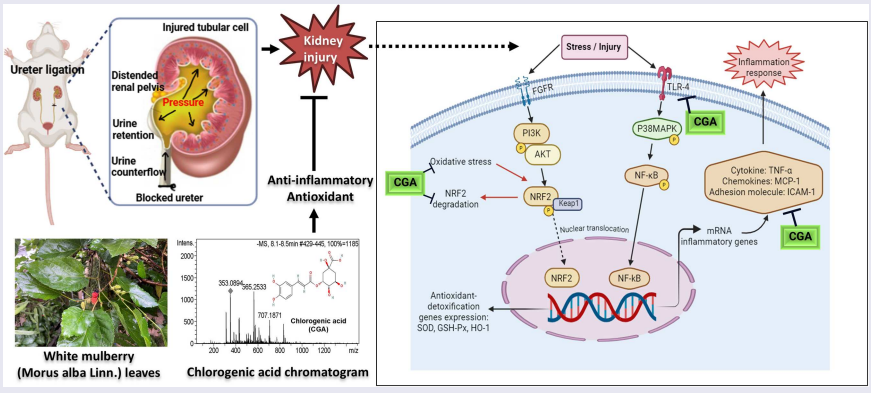
ABSTRACT

White mulberry leaf extract (WME) is known for its anti-inflammatory and antioxidative stress properties. Nevertheless, the mechanisms by which WME and its primary compound mitigate kidney damage in obstructive nephropathy remain unclear. This study investigates the efficacy of WME and chlorogenic acid (CGA) in suppressing inflammation and oxidative stress in animal models of obstructive nephropathy. Male Balb/c mice ($n = 25$) were randomly distributed into five groups: (1) the sham operation (SO) group, (2) the unilateral ureter obstruction (UUO) group, and UUO-induced mice treated with (3) enalapril (ENA), (4) WME, and (5) CGA. After the experiment, kidney organs were isolated for gene and protein analyses. Pro-inflammatory genes, including TLR-4, ICAM-1, and MCP-1, were measured using qPCR. The protein expression of TNF-*α* and NRF2 was examined using immunohistochemistry. The expression of TLR-4, ICAM1, and MCP-1 was downregulated in the WME and CGA groups ($p < 0.0001$) compared with the UUO group. TNF-*α* expression was markedly lower in the WME and CGA groups ($p < 0.05$) than in the UUO group. Furthermore, increased NRF2 protein expression demonstrated improved oxidative stress in the WME and CGA groups ($p < 0.05$). These findings indicate that WME and CGA mitigate kidney damage in obstructive nephropathy.



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1. Introduction

Chronic kidney disease (CKD) is a progressive disease that causes high mortality and morbidity, and individuals with high blood pressure and diabetes have a higher incidence (Kalantar et al. 2021). Furthermore, kidney fibrosis is the hallmark pathological alteration observed in CKD (Bülow and Boor 2019). In murine animals, unilateral ureteral obstruction is a well-established mechanism for developing obstructive nephropathy associated with kidney fibrosis (Mart et al. 2019). In progressive renal fibrosis, inhibiting inflammation and oxidative stress is crucial for preventing CKD (Hu et al. 2021). Research indicates that early intervention in acute renal injury stages can lead to the recovery of the organ's structure and function, effectively preventing chronic complications and restoring its functionality (Chawla et al. 2014). Meanwhile, in situations involving chronic inflammation, persistent cytokine and chemokine production inhibits normal healing and promotes the rapid progression of renal damage (Zeisberg and Neilson 2010). Several inflammatory responses, including TNF-α, TLR-4, ICAM1 and MCP-1, have been reported to be involved in kidney injury or tubulointerstitial fibrosis (Ren et al. 2024). These cytokines play a pivotal role in CKD development. For instance, a study by Mazur et al. noted an increase in TNF-α concentrations, alongside other inflammatory mediators, in patients with CKD, indicating its role in disease progression (Mazur 2021). Moreover, there is evidence suggesting that TNF-α can stimulate the production of MCP-1, thereby creating a feedback loop that exacerbates renal inflammation (Borza 2022). Likewise, Liu et al. highlighted that MCP-1 is a critical mediator of renal inflammation by promoting monocyte infiltration (Liu 2024). Additionally, Chang et al. reported that ICAM-1 expression can be upregulated in response to oxidative stress, further contributing to renal inflammation and fibrosis (Chang 2024). Thus, targeting these cytokines for therapeutic intervention could be aimed at modulating inflammation and mitigating CKD progression.

The white mulberry plant (*Morus alba* Linn.) grows in tropical conditions and is commonly found in Southeast Asian countries (Rynko et al. 2016). The utilization of mulberry leaves as herbal medicine has shown significant growth in recent decades, emerging as a popular alternative therapy (Ji et al. 2021). In traditional Chinese medicine, *Morus alba* Linn. plant alleviates fever and cold symptoms (Zhang et al. 2022). Its biological activities include anti-inflammatory, antioxidant, hepatoprotective, cardioprotective and antidiabetic properties (Memete et al. 2022). Notably, the leaves of mulberries are abundant in bioactive chemicals, particularly flavonoids, which possess strong antioxidative activities (Yadav et al. 2022). Studies have suggested that flavonoids have the potential to effectively inhibit oxidative stress (Doi et al. 2000) and reduce inflammatory responses (Cao et al. 2022). Moreover, mulberry leaves also possess nephroprotective properties and may help reduce renal damage (Nematbakhsh et al. 2013; Rebai et al. 2021). Our previous study demonstrated that chlorogenic acid (CGA) is the primary compound in white mulberry leaves (Fauzi et al. 2024). Nevertheless, the mechanism by which CGA affects kidney damage remains restricted, and the specific mechanism regulating pro-inflammatory responses require further elucidated. Thus, the objective of the current study was to determine the anti-inflammatory and antioxidative properties of white mulberry leaf extract and CGA in obstructive nephropathy-induced mice.

2. Materials and methods

2.1. White mulberry leaf ethanolic extraction procedure

White mulberry leaves harvested from Selangor, Malaysia, were thoroughly cleaned with fresh water to remove dirt and dust adhering to their surfaces. The leaves were dehydrated using an oven set at 50 °C for 12 h. Subsequently, the leaves were pulverized into a fine powder and preserved at 4 °C for subsequent processing. A total of 1500 mL of a 60% ethanol-water solution was used to dissolve 100 g of leaf powder, following a modified method of Zhou et al. (2022) to obtain high phenolic and flavonoid contents in the leaves. The extract was concentrated using a rotary evaporator after being filtered through No. 1 filter paper. The concentrated extract was stored in a refrigerator at 4 °C for subsequent analysis.

2.2. Determining phenolic compounds of WME using liquid chromatography–mass spectrometry (LC–MS)

Approximately 1 mg of the concentrated WME was measured and added to 1 mL of LC–MS grade methanol. The solution was homogenized using a vortex (Corning LSE 6776, Taiwan) for 10 min to break down the solute fully. The extracted sample solution was passed through a Sartorius minisart filter (0.45 µm pore size)

before being transferred into an LC–MS vial. The polyphenol content of WME was evaluated using LC–MS instruments equipped with an UltiMate 3000 series and a Bruker microTOF-QIII mass spectrometer. Putative compounds were identified in WME using MetFrag software, and the results were validated by Dr. Ilya Mahmod, a certified chemist from the Nutrition Research Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

2.3. Experimental animal

Animal experiments were conducted in accordance with the experimental protocol approved by the University of Putra Malaysia's Ethical Clearance committee, with registration number 2022/AUP-R024. Male Balb/C strain mice, 8 weeks old and weighing 25 ± 2 g, were purchased from the Laboratory Animal Facility and Management at the Faculty of Pharmacy, UiTM, Malaysia. Animals were housed in transparent polycarbonate cages (320 x 180 x 150 mm) at the BSL-1 animal research facility, Faculty of Veterinary Medicine, UPM, at 23–25 °C and 50%–60% humidity, with alternating 12-h light–dark cycles. The air exchange rate in the animal facility was maintained at 6–10 air changes per hour. Mice were fed standard murine chow (Gold Coin Holding, Kuala Lumpur, Malaysia) and given fresh water ad libitum. A kidney fibrosis model was established by ligating the unilateral ureter for 2 weeks. UUO surgery was performed according to Hesketh et al. (2014). They were anaesthetized using a drug mixture of ketamine 100 mg/kg bw (Ket-A-100®, Agrovet, Peru), xylazine 10 mg/kg bw (Xyla®, Interchemie, Holland), and acepromazine 2 mg/kg bw (Castran®, Interchemie, Holland) injected intraperitoneally, followed by cardiac puncture for euthanasia. A total of twenty-five mice of experimental animals were randomly assigned into five groups: (1) baseline control group, known as sham operation (SO); (2) negative control group, known as unilateral ureteral obstruction (UUO); (3) positive control group, UUO-induced mice and received 15 mg/kg enalapril (ENA); (4) the UUO-induced mice and received 125 mg/kg WME; and (5) a group of UUO-induced mice and received 25 mg/kg CGA. The animals received oral medication once daily for 14 days, starting on the second day after UUO induction. At the end of the investigation, mice were sacrificed, and the kidneys were collected for further analysis. Half of the obstructed kidney was fixed in 10% buffered formalin and paraffin embedded was then performed for immunohistological staining. The residual kidney tissue was placed in RNAlater solution for further RNA extraction.

2.4. Experimental medication and dosage regiment

In this study, the positive control group received 15 mg/kg Enapril® (enalapril maleate, produced by YSP Industries, Malaysia) via oral gavage as a standard pharmacotherapy for renal disease. Meanwhile, 125 mg/kg of WME, dissolved in 5% dimethyl sulfoxide (DMSO) solvent was given by oral gavage to the mice, the extract dosage was selected based on a toxicity study in the previous investigation (Fauzi et al. 2024). A total of 25 mg/kg chlorogenic acid hydrate (98.0% pure compound, provided by Tokyo Chemical Industry, Japan) was administered to mice via oral gavage. The dosage was adapted from a previous mouse investigation (Owumi et al. 2021). The sham operation involved the administration of 10 mL/kg of 5% DMSO via oral gavage.

2.5. Real-time-quantitative polymerase chain reaction (RT-qPCR)

Upon completion of the experiment, kidneys were separated for quantitative real-time polymerase chain reaction (qPCR). The manufacturer's instructions were followed to extract the kidney tissue's total RNA using Roche Diagnostics GmbH's high-purity RNA tissue kit. The mRNA expression of monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and toll-like receptor 4 (TLR-4) was analyzed to determine the expression of pro-inflammatory response. The primer sequences of pro-inflammatory genes were customized using the Primerquest™ tool by Integrated DNA Technology (IDT) (Table 1). Synthesis of cDNA from RNA samples using the QuantiTech Reverse Transcription Kit (Qiagen, Germany) per the manufacturer's instructions. The qPCR analysis was conducted using GoTaq qPCR Master Mix (Promega Ltd., UK). The Applied Biosystems™ StepOne™ Real-Time PCR System (Poster City, CA, U.S.A.) was used to establish a qPCR program with standard cycle conditions: 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60–64 °C for 1 min, and 95 °C for 15 s. The results quantification was analysed using ΔΔCt following the Pfaffl method.

Table 1. The primer sequences of the pro-inflammatory genes utilized for the RT-qPCR reaction.

No	Inflammatory genes	Genbank accession ID	Primer sequence
1	Toll-like receptor-4 (TLR-4)	NM_021297.3	Forward 5′-CAGCCAGATAGCACAGAAGAG-3′ Reverse 5′-GACTGGCACTAACCACATAGAG-3′
2	Monocyte chemoattractant protein-1 (MCP-1)	NM_011333.3	Forward 5′-AGTAGGCTGGAGAGCTACAA-3′ Reverse 5′-GTATGTCTGGACCCATTCCTTC-3′
3	Intercellular adhesion molecule-1 (ICAM-1)	NM_010493.3	Forward 5′-GATGCTCAGGTATCCATCCATC-3′ Reverse 5′-CCACTCTCGAGCTCATCTTTC-3′
4	Beta-actin (ACTB)	NM_007393.5	Forward 5′-CTCCCTGGAGAAGAGCTATGA-3′ Reverse 5′-CACAGGATTCCATACCCAAGAA-3′

2.6. Immunohistochemistry of TNF-α and NRF-2

Paraffin-embedded kidneys were deparaffinized with xylene, rehydrated with a gradient alcohol series, and antigen retrieved with 3% (v/v) H_2O_2 following blocking with 3% BSA and 0.25% Triton X-100 blocking buffers. The sections were subsequently incubated with primary antibody against mouse tumor necrosis factor-alpha (TNF-*α*, sc-52746, Santa Cruz Biotechnology, CA, U.S.A.) and nuclear factor-erythroid-2 related factor 2 (NRF2, bs-2013R, Bioss Inc., Massachusetts, U.S.A.) at 4 °C overnight. Conjugated horseradish peroxidase of universal antibodies was used as the secondary antibody and was incubated for an hour at room temperature. After being washed with PBS, the sections were stained with 3,3-diaminobenzidine or DAB and counterstained with Meyer's haematoxylin. The slides were examined using a light microscope (80i Nikon Eclipse, Tokyo, Japan) and photographed with an electronic camera (DS-Ri1, Nikon, Tokyo, Japan).

2.7. Semi-quantitative determination of TNF-α and NRF-2

Semi-quantitative analysis of TNF-*α* and NRF-2 expression was quantified using the ImageJ program (National Institutes of Health, U.S.A.). Ten images from each slide were captured and quantified following the Gewehr (2021) method. DAB-stained areas with brown histological staining were considered positive. TNF-*α* and NRF-2 protein expression levels were determined according to the percentage of positive area intensity (Crowe and Yue 2019).

2.8. Statistical analysis

Data analysis was performed using SPSS Statistics 26.0 program, and graphics were drawn utilizing GraphPad Prism 9.0 (GraphPad Prism, California, U.S.A.). Group comparisons were assessed using one-way analysis of variance (ANOVA) and Tukey's post-hoc test, as the data were normally distributed. The average values are presented as the standard error of the mean (SEM). *P*-values that were less than or equal to 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Phytochemicals content of white mulberry leaf extract

White mulberry leaves contain various nutrients and bioactive compounds. Following the LC–MS identification of the present study, the phytochemicals in WME included as many as 20 compounds with negative and positive ionization (Figure 1). Among the 20 phytochemical compounds identified, 11 flavonoids, 5 phenolic acids and 4 benzofurans were classified. Flavonoids were the predominant polyphenolic class of compounds discovered in WME, followed by phenolic acids and benzofurans. The flavonoids detected in WME were kaempferol-3,7-glucopyranoside, euchrenone A, rutin, glycitin, 3'-hydroxy daidzein, hydroxytyrosol-4-O-glucoside, morkotin A, multinoside A, isoquercitrin, kaempferol 3-O-rhamnoside-7-O-glucoside and astragalin. The phenolic acid class of compounds includes quinic acid, chlorogenic acid, neochlorogenic acid, 3,4-DHPEA-AC and 5-O-*p*-coumaroylquinic acid. The benzofuran class of compounds consists of moracin C, moracin N, chalcomoracin and mulberroside F.

Furthermore, chlorogenic acid was established as one of the primary compounds of white mulberry leaves. Our previous study showed that the chlorogenic acid content in white mulberry leaves, using the analytical technique of HPLC quantification, was 306 mg/100 g dry weight (Fauzi et al. 2024). Figure 2

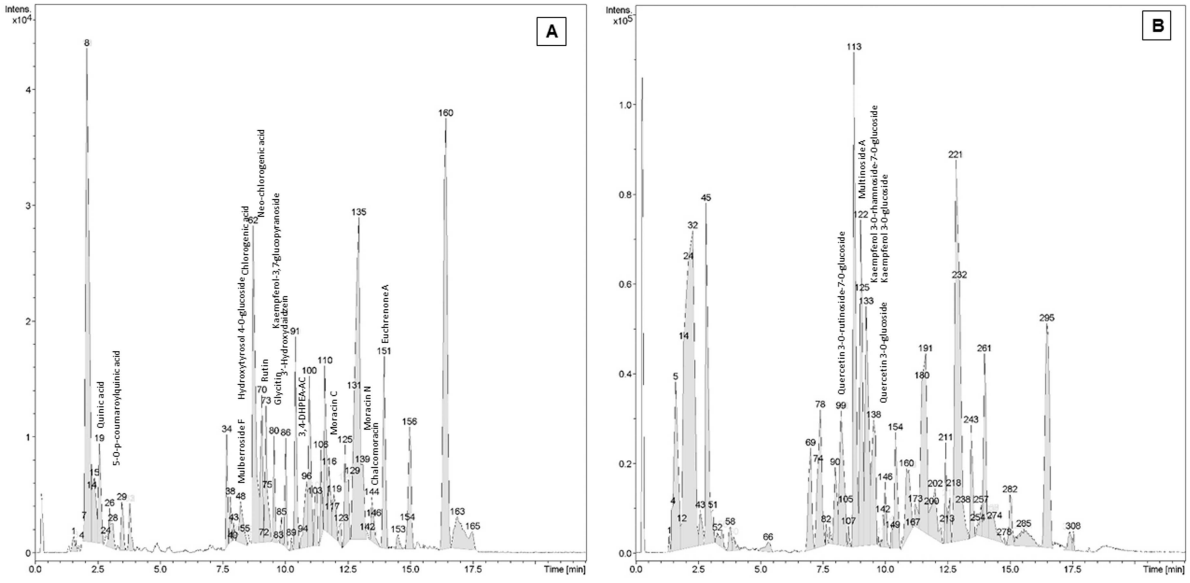

Figure 1. LC–MS of WME in negative mode ionization (A) and positive mode ionization (B).

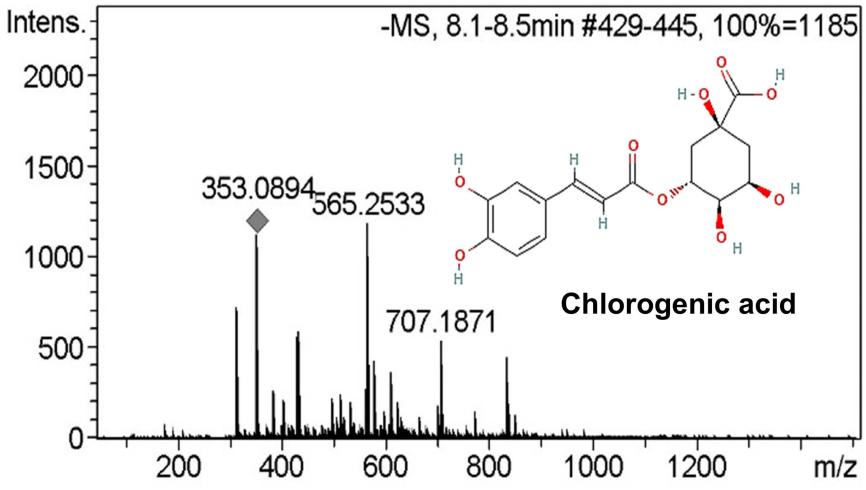

Figure 2. Chromatogram of chlorogenic acid contained in the WME.

illustrates the chromatogram of the chlorogenic acid contained in the white mulberry leaf extract, using the analytical technique of LC–MS compound identification.

3.2. The effect of enalapril, white mulberry leaf extract and chlorogenic acid on suppressing the pro-inflammatory response of TLR-4, MCP-1 and ICAM-1 gene expression in obstructive nephropathy-induced mice

The pro-inflammatory gene expression quantified using the real-time qPCR is presented in Figure 3. The expression of TLR-4, a receptor that stimulates cytokine and chemokine production, was significantly greater ($p < 0.0001$) in the UUO group compared to the SO group (Figure 3A). On the other hand, enalapril treatment dramatically reduced TLR-4 expression ($p < 0.0001$) in UUO-induced animals. Compared with the UUO group, the WME and CGA groups presented significantly lower TLR-4 expression ($p < 0.0001$), suggesting the capacity of these groups to suppress proinflammatory receptors. In addition, the UUO group presented significantly higher expression of MCP-1 ($p < 0.0001$), a gene that attracts monocytes and

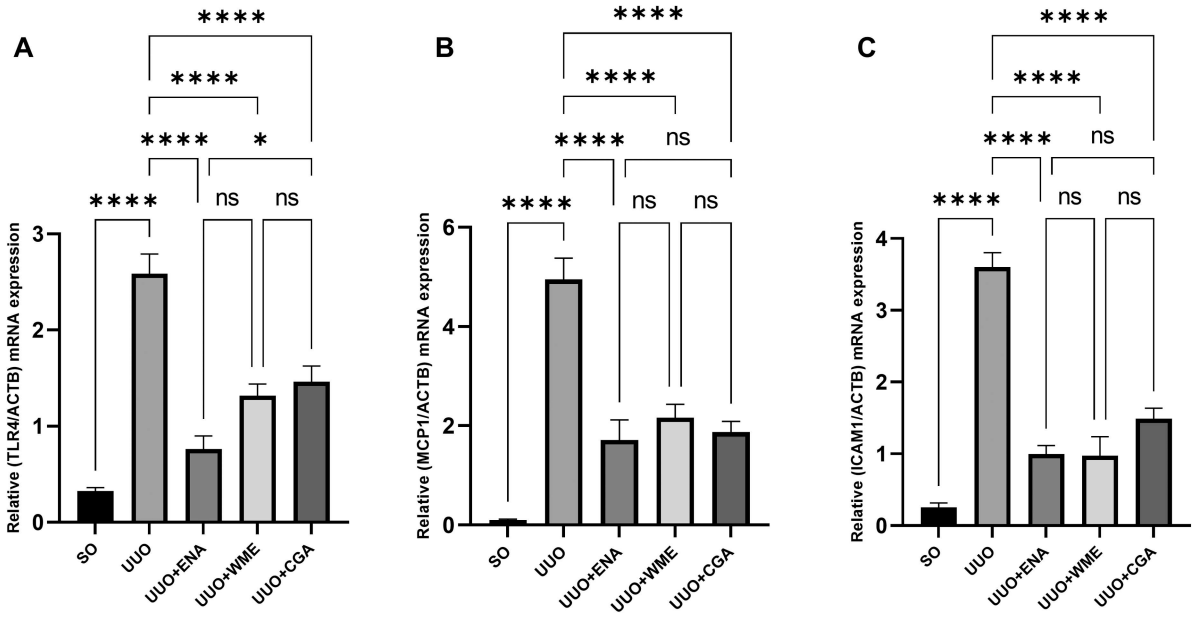

Figure 3. Quantitative mRNA expression of MCP-1 (A), ICAM-1 (B) and TLR-4 (C) in the kidneys of mice with unilateral ureteral obstruction-induced mice and treated with enalapril, white mulberry leaf extract and chlorogenic acid. The bar value represents the mean ± SEM; key: SO = Sham operation; UUO = unilateral ureteral obstruction; ENA = enalapril; WME = white mulberry leaf extract; CGA = chlorogenic acid. Key ns: not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

macrophages to induce inflammation (Figure 3B). In contrast to that in the UUO group, MCP-1 expression in the UUO-induced kidney fibrosis group was significantly reduced ($P < 0.0001$) by treating WME and CGA. The outcome was comparable to that of enalapril treatment as the control medication, suggesting that these compounds may reduce the attraction of inflammatory cells. Furthermore, the UUO group exhibited substantially higher expression of ICAM-1, an adhesion receptor gene that regulates leukocyte recruitment, compared to the SO group ($p < 0.0001$) (Figure 3C). ICAM-1 expression in the ENA, WME and CGA groups was markedly downregulated compared to the UUO group ($p < 0.0001$), indicating the capacity of WME and CGA treatments to prevent inflammation by inhibiting ICAM-1 expression.

3.3. The effect of enalapril, white mulberry leaf extract and chlorogenic acid on inhibiting the pro-inflammatory cytokine TNF-α and oxidative damage of NRF-2 protein expression in obstructive nephropathy-induced mice

The immunohistochemical labelling of TNF-*α* protein expression in the kidneys of the UUO group is illustrated in Figure 4. Compared to the other groups, the UUO group exhibited remarkable expression of TNF-*α* in the renal tubule region, as expected. These results aligned with the pro-inflammatory gene expression in the kidney, indicating that untreated UUO-induced mice promote the expression of inflammation-related genes and proteins. The ImageJ program was subsequently used to evaluate the intensity of TNF-*α* expression via immunohistochemistry (Figure 5). The quantification results generally revealed that the SO group presented the lowest TNF-*α* expression compared to the other groups, with an area intensity of 1.12% ± 0.10.

In contrast to the SO group, the UUO group exhibited a higher level of TNF-*α* expression ($p < 0.05$), with an area intensity of 34.44% ± 1.73. Conversely, the ENA group exhibited a 12.04% ± 1.71 area-intensity reduction in TNF-*α* expression ($p < 0.05$) compared with the UUO group. Moreover, the WME group demonstrated a significant decrease ($p < 0.05$) in TNF-*α* expression compared to the UUO group, with an area intensity of 15.47% ± 2.24. Finally, the CGA group exhibited a significant decrease in TNF-*α* expression ($p < 0.05$) compared to the UUO group, with an area intensity of 10.56% ± 2.20.

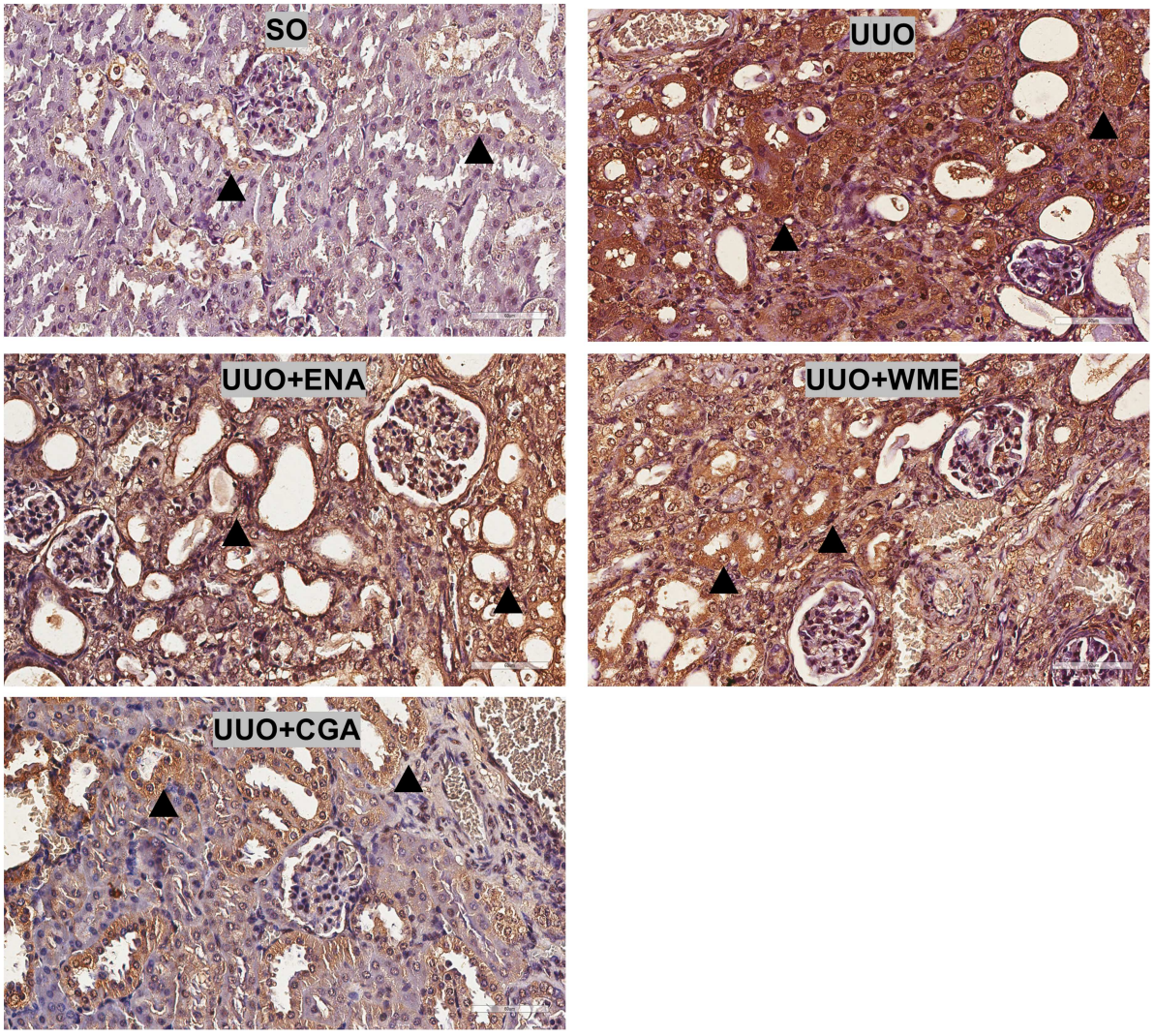

Figure 4. Representative light photomicrograph of TNF-*α* immunohistochemistry and semi-quantitative TNF-*α* expression in UUO-induced mice's kidney (200x magnification). Untreated UUO-induced mice depicted increased TNF-*α* expression in tubules (black arrowhead). However, UUO-induced mice treated with enalapril, white mulberry leaf extract and chlorogenic acid presented lower TNF-*α* expression levels than the UUO group.

The immunohistochemical labelling of NRF-2 protein expression in the mice kidneys is illustrated in Figure 6. Unsurprisingly, the SO group presented the highest NRF-2 expression among the groups. Meanwhile, the UUO group showed significantly lower NRF-2 expression in the renal tubule region than the SO group, indicating that the obstructive nephropathy-induced mice could not maintain the cellular redox balance due to increased renal inflammation and oxidative stress. However, enalapril, WME and CGA treatment demonstrated an increased NRF-2 expression compared to the UUO group.

Furthermore, the ImageJ program was employed to evaluate the intensity of NRF-2 expression in the immunohistochemistry (Figure 7). Briefly, the quantification results indicate that the SO group exhibited the highest NRF-2 expression compared to the other groups, with an area intensity of 19.54% ± 1.89. The UUO group demonstrated a lower level of NRF-2 expression ($p < 0.05$) than the SO group, with an area intensity of 4.97% ± 1.10.

Conversely, the ENA group showed a rise in area intensity of 15.19% ± 1.39 in NRF-2 expression ($p < 0.05$) in comparison to the UUO group. Conversely, the WME groups (15.22% ± 1.65) exhibited a significantly higher level of NRF-2 expression ($p < 0.05$) than the UUO group. Finally, the CGA group demonstrated a substantial increase in NRF-2 expression ($p < 0.05$) compared to the UUO group, with an area intensity of 14.64% ± 1.08.

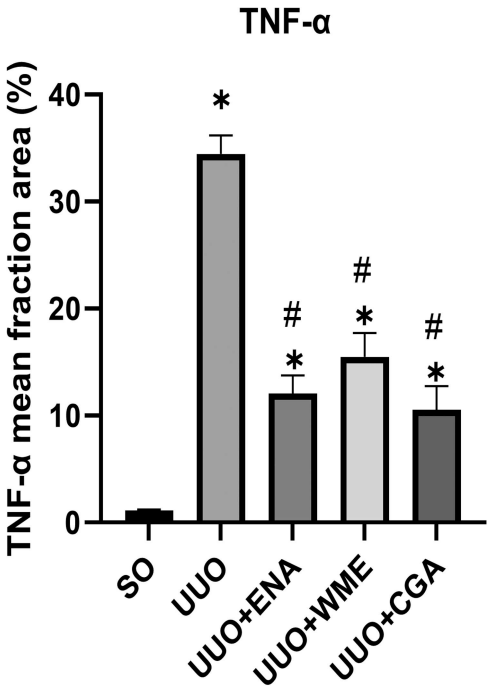

Figure 5. Semi-quantitative assessment of the TNF-*α* expression area of the mice kidney in different groups using image-J software. Significance: $^{*}p < 0.05$ *vs. SO group;* $^{\#}p < 0.05$ vs. UUO group.

4. Discussion

It is well established that TLR-4 is a critical receptor involved in mediating the inflammatory response in various acute and chronic diseases (Sidletskaya et al. 2020). Upon ligand binding, TLR-4 recruits MyD88 adaptor proteins, leading to the activation of NF-κB and mitogen-activated protein kinases (MAPKs) (Nighot et al. 2015). This signalling cascade results in the upregulation of TNF-*α*, which amplifies the inflammatory response by promoting immune cell recruitment (Laha and Grant, 2021). Simultaneously, MCP-1 enhances the chemotactic migration of monocytes/macrophages into the renal interstitium, contributing to inflammation and tissue remodelling (Singh et al. 2021). Additionally, ICAM-1 expression on endothelial and tubular epithelial cells facilitates leukocyte adhesion and transmigration, further exacerbating renal injury (Oates et al. 2022). In line with previous investigations, the present study found that UUO-induced mice presented increased TLR-4 gene expression, which triggered inflammatory responses by upregulating TNF-*α* cytokines, MCP-1 chemokines and ICAM-1 adhesion molecules, as shown by the RT-qPCR results. Meanwhile, WME administration significantly reduced TLR-4 expression in the UUO-induced mice kidney, consistent with a previous study (Hung 2023). In addition, WME treatment effectively downregulated key pro-inflammatory mediators, including TNF-*α*, MCP-1 and ICAM-1, which is in agreement with earlier studies conducted in diabetic nephropathy animal models (Gurukar and Chilkunda 2018) and LPS-stimulated RAW 264.7 macrophage cells (Lin 2022). Furthermore, WME also demonstrated antioxidant properties by preserving NRF2-mediated redox homeostasis in UUO-induced mice, supporting previous observations that highlighted the extract's role in combating oxidative stress (Tang 2023). The capacity of white mulberry leaf to limit oxidative stress release may be attributed to its abundant flavonoids and phenolic acids, which are responsible for counteracting inflammatory reactions (Lin 2022). A study showed that flavonoids in white mulberry leaves mitigated oxidative stress by suppressing phosphatidylinositol 3-kinase (PI3K)/Akt and maintaining NRF2 as an intracellular antioxidant (Ko 2021). Phenolic compounds can modulate inflammation-related signalling pathways, including nuclear factor (NF)-κB, MAPKs, phosphatidylinositol 3-kinase (PI3K)/Akt and the ubiquitin-proteasome system (Costa et al. 2012; Rojas-García et al. 2023).

Furthermore, it is well known that mulberry leaves contain many polyphenols that have antioxidant and anti-inflammatory properties (Leyva-Jiménez 2020). Based on the LC–MS analysis, this study identified that the mulberry leaf extract contains several flavonoid chemicals, such as quercetin, kaempferol and other derivatives, which have been recognized as potential inhibitors of inflammation (Lin 2022). For instance,

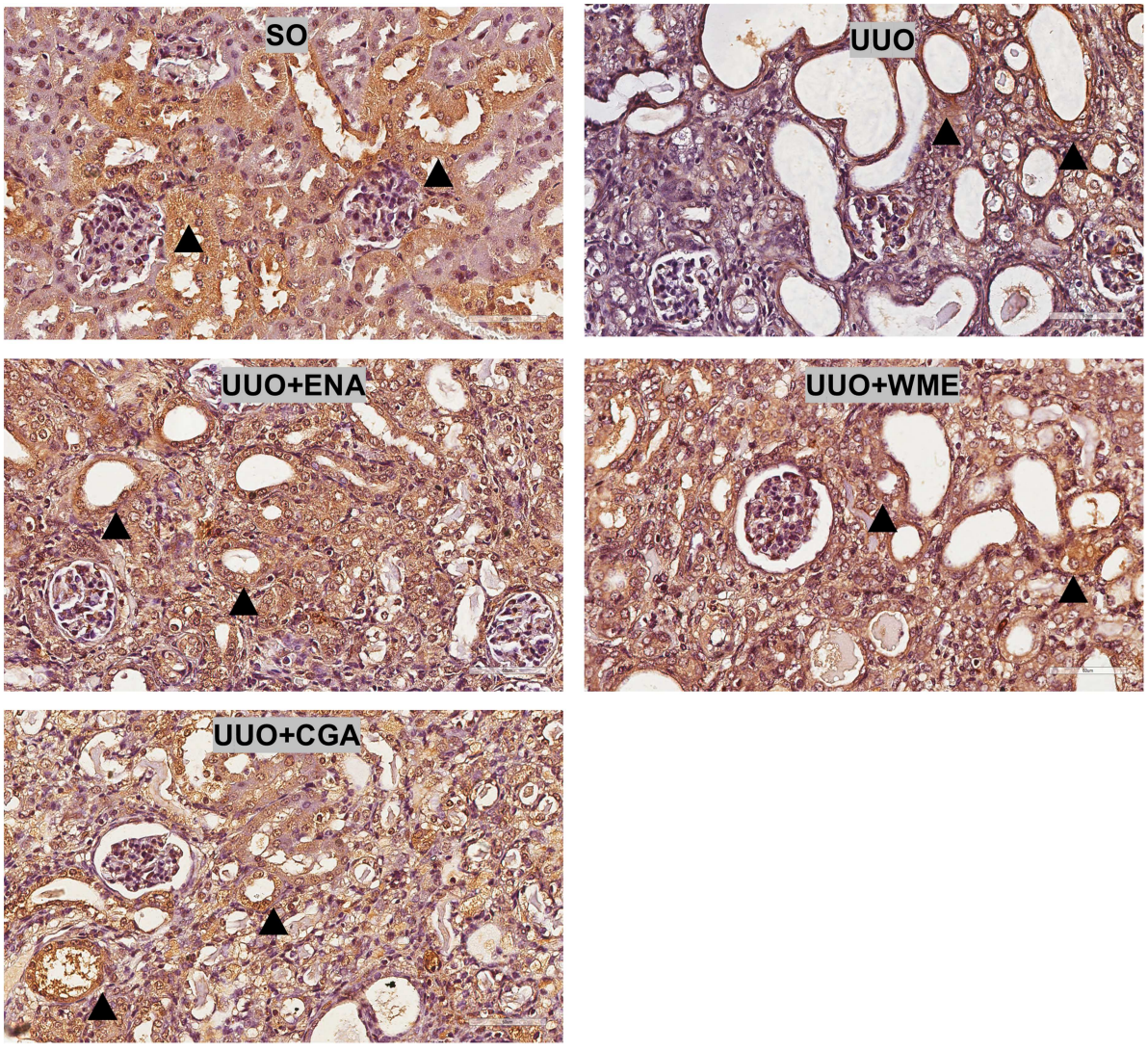

Figure 6. Representative light photomicrograph of NRF-2 immunohistochemistry and semi-quantitative analysis of NRF2 expression in the kidney of UUO-induced mice (200x magnification). Untreated UUO-induced renal fibrosis mice depicted decreased NRF-2 expression in tubules (black arrowhead). However, UUO-induced mice treated with enalapril, white mulberry leaf extract and chlorogenic acid presented increased the level of NRF-2 expression compared to the UUO group.

quercetin can improve kidney damage by regulating the inflammatory response in M1/M2 macrophages in UUO-induced obstructive mice (Lu 2018). Similarly, kaempferol has been shown to enhance the activation of NRF2 and HO-1 (Alshehri 2023) and reduce the inflammatory responses (Luo 2021).

Moreover, the LC–MS results revealed that flavonoid compounds were more dominant than phenolic acids and benzofurans. Nonetheless, additional research has suggested that chlorogenic acid and neo-chlorogenic acid are among the phenolic chemicals in mulberry leaf extract capable of modulating inflammation (Gao et al. 2020). These compounds have been shown to inhibit NF-κB, TNF-α, IL-6, and apoptotic markers Bax and Bcl2 in endoplasmic reticulum stress-induced mice (Moslehi et al. 2023). The present study revealed that chlorogenic acid administration in UUO-induced mice reduced the pro-inflammatory response and oxidative stress. Quantitative PCR analysis indicated that CGA decreased the expression of TLR-4, MCP-1 and ICAM-1 in UUO-induced mice, which aligns with prior studies (Arfian 2019; Bisht et al. 2020). Moreover, a previous study reported that chlorogenic acid suppressed inflammation by inhibiting the nuclear factor-kB (NF-kB)/TLR-4 pathway, which reduces the release of IL-1β, IL-6 and TNF-*α* (Yu et al. 2021). In line with these findings, the current study demonstrated that CGA treatment in UUO-induced mice reduced TNF-*α* expression, indicating its ability to reduce inflammation in kidney tissue. Besides that, gene analysis also revealed that CGA decreased ICAM-1

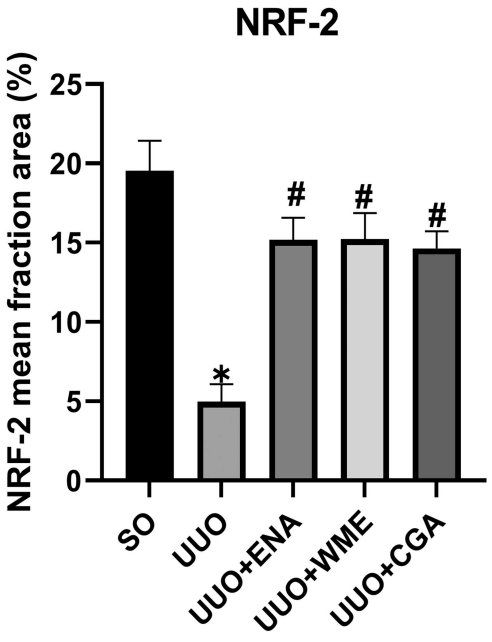

Figure 7. Semi-quantitative assessment of the NRF-2 expression area of the kidney mice in different groups was performed using image-J software. Significance: $^{*}p < 0.05$ vs. SO group; $^{\#}p < 0.05$ vs. UUO group.

expression, which supported these findings. CGA downregulates the gene and protein expression of adhesion molecules, including ICAM-1, VCAM-1 and ELAM-1, in mice-induced hepatitis (Yuan et al. 2017). Furthermore, chlorogenic acid treatment in UUO-induced mice enhanced the cellular antioxidant response by regulating NRF2 expression in kidney tissue, consistent with a previous investigation (Liu et al. 2020). A previous study reported that chlorogenic acid suppresses oxidative damage by inhibiting cyclooxygenase-2 (COX-2) and stimulating heme oxygenase-1 (HO-1) and cytochrome P450 E1 (CYP2E1) and 4-hydroxynonenal (4-HNE) (Domitrovi et al. 2014). Chlorogenic acid possesses anti-inflammatory and antioxidant properties, which may be due to its hydrolytic activity within the body. According to one study, chlorogenic acid undergoes hydrolysis upon entering the digestive tract, where it forms three small compounds: quinic acid, ferulic acid and caffeic acid (Naveed 2018). These compounds reduce organ inflammation and act as antioxidant agents (Kadoma and Fujisawa 2008; Damasceno et al. 2017).

This study demonstrated that white mulberry leaf extract or chlorogenic acid treatment maintained the cellular antioxidant NRF2 and inhibited the inflammatory response in obstructed nephropathy-induced mice. A summary of how white mulberry leaf extract and chlorogenic acid inhibit kidney inflammation and oxidative stress in obstructed nephropathy mice is presented in Figure 8. It is noteworthy that WME and CGA had the same impact on reducing inflammation in UUO-induced rodents as enalapril, a standard medication employed to treat CKD. Enalapril administration in UUO-induced mice significantly decreased the expression of TLR-4, MCP-1, ICAM-1 and TNF-α, which was consistent with the previous findings (Li et al. 2020; Bryniarski et al. 2022; Krzemińska and Wronka, 2022). These findings also demonstrated that enalapril maintains the cellular antioxidant in UUO-induced mice, as represented by increasing NRF2 expression. Enalapril is an angiotensin-converting enzyme (ACE) inhibitor, which lowers the synthesis of angiotensin II, the main effector peptide in the renin–angiotensin system (Bryniarski et al. 2022). Angiotensin II is a potent mediator of pro-inflammatory and pro-fibrotic effects in the kidney, largely by activating oxidative stress-related pathways, inducing pro-inflammatory cytokines, and enhancing the deposition of extracellular matrix (ECM) (Azushima 2019; AlQudah et al. 2020). These processes are particularly relevant in pathological conditions such as UUO, where sustained elevation of angiotensin II exacerbates tubulointerstitial inflammation and fibrosis (Zhang et al. 2021). Enalapril may exert renoprotective effects by blocking the formation of angiotensin II, and it ameliorates inflammation and decreases oxidative stress (Cozzoli 2011). Taken together, these findings collectively indicate the potential of WME, which contains chlorogenic acid, as an anti-inflammatory and antioxidant, similar to the action of enalapril.

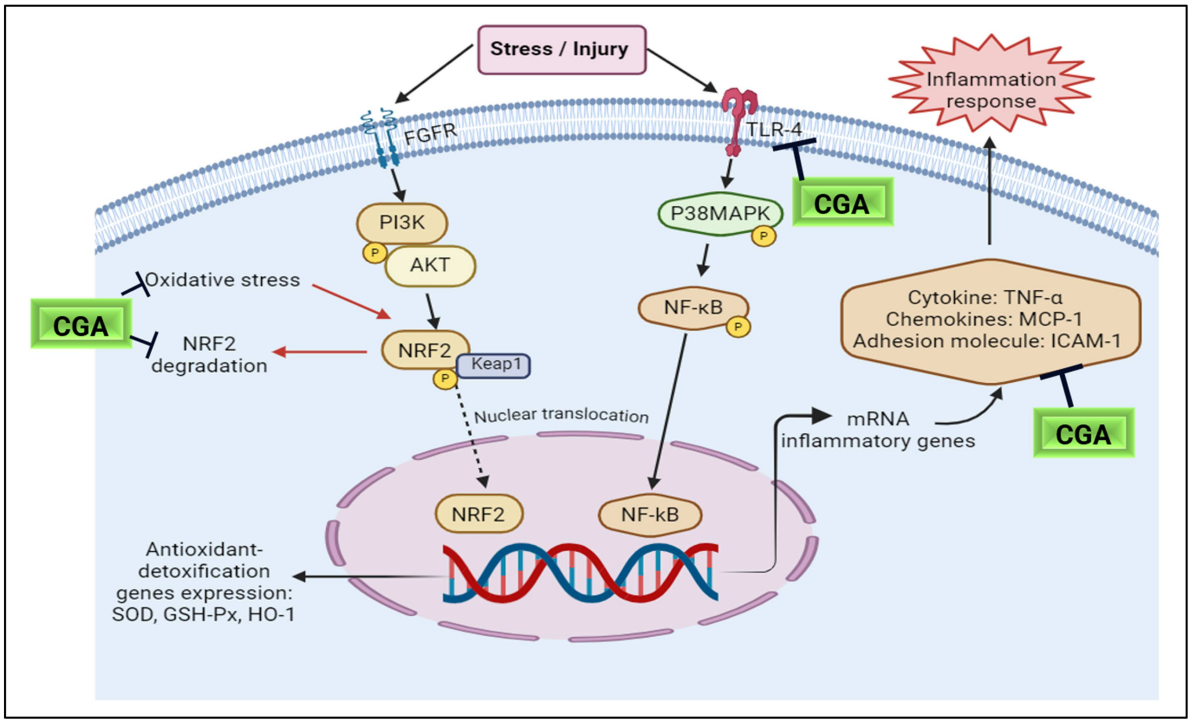

Figure 8. Regulation of the NRF2 and TLR-4/TNF-α/MCP-1/ICAM-1 signalling pathways by chlorogenic acid upregulated antioxidant activity and suppressed inflammation. Stress stimuli and kidney injury will activate the signalling receptor of TLR-4 to engage and phosphorylate the inflammatory signalling response of p38MAPK and NF-κB. NF-κB phosphorylated translocate to the nucleus and promotes the transcription of NF-κB-dependent genes of cytokine, chemokine and adhesion molecule, including TNF-*α*, MCP-1 and ICAM-1. The upregulated inflammatory genes stimulate an inflammation response in cells. In addition, phosphorylation of PI3K and AKT modulates the NRF2/Keap1 signalling pathway, in which oxidative stress causes cleavage of NRF2 and Keap1. NRF2 ceases to undergo degradation in the proteasome and is transported into the nucleus, which leads to the activation of antioxidant response element (ARE)-containing genes, including SOD, GSH-Px and HO-1. According to the findings, white mulberry leaf extract, which includes chlorogenic acid, mitigates kidney alterations by modulating proinflammatory cytokines and oxidative stress.

This study is limited by its focus on a single animal model (UUO-induced kidney fibrosis), which may not completely capture the complexity of chronic kidney diseases in humans. Additionally, long-term follow-up to evaluate its protective effects is lacking. Despite these limitations, WME downregulates TLR-4 and pro-inflammatory-related genes, demonstrating its dual anti-inflammatory and antioxidant properties. This study contributes to the expanding body of evidence regarding the therapeutic potential of mulberry-derived bioactive compounds in renal pathophysiology by employing RT-qPCR for gene expression analysis. The study addresses multiple mechanistic pathways, making it more comprehensive. Therefore, future research should include more models, long-term assessments, and biomarker profiling to enhance findings, including parameters related to antioxidants (SOD, GPx, GSH or ROS) and cytokines (IL-6 and IL-*β*), to strengthen the research.

5. Conclusion

In conclusion, the study demonstrated that white mulberry leaf extract and chlorogenic acid inhibited the inflammatory response in obstructive nephropathy by suppressing the expression of TLR-4, MCP-1, ICAM-1 and TNF-*α*, and by modulating the intracellular antioxidant activity of NRF2. Thus, it could potentially be a therapeutic option for preventing the progression of renal disease.

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Author contributions

Ahmad Fauzi: Experimentation, data curation and analysis, writing (original draft); Nurina Titisari: Editing and data curation; Mohd. Hezmee Mohd. Noor: Editing and supervision; Azrina Azlan: funding acquisition, project administration and supervision; Hazilawati Hamzah: conceptualization, validation, review and editing.

Disclosure statement

The authors declare that there is no conflict of interest.

Data availability statement

Data will be made available on request.

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