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ORIGINAL RESEARCH

Effects of Zofenopril and Thymoquinone in Cyclophosphamide-Induced Urotoxicity and Nephrotoxicity in Rats; The Value of Their Anti-Inflammatory and Antioxidant Properties

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Objective: The study aimed to investigate whether zofenopril (ZOF), thymoquinone (TQ), or their co-administration effectively ameliorates urotoxicity and nephrotoxicity following cyclophosphamide (CPH) treatment.

Methods: A total of 48 Wister Albino female rats were divided into six groups each of eight rats; negative control (NC), positive control (PC), mesna (MS), ZOF, TQ, and ZOF+TQ groups. Normal saline, mesna, ZOF-15mg/kg, TQ-80mg/kg, and their combination were given orally for 19 days to the groups NC, MS, ZOF, TQ, and ZOF+TQ respectively. On the 17th day, a single dose of CPH 200 mg/kg was given intraperitoneally for all the groups except the NC group. Urine was collected over 24 hours before animal scarification for urinalysis. After scarification, blood, and kidney tissue were obtained for assessment of conventional kidney function parameters, novel kidney injury biomarkers, pro-inflammatory cytokines, oxidative status, complete blood count (CBC), and histopathological examination.

Results: CPH disturbed the urinary excretion of urea, creatinine, and protein, and significantly elevated novel biomarkers for kidney injury including cystatin-C (Cys-C) (p=0.019) and markedly kidney injury molecule-1 (KIM-1) (p=0.27), the semiquantitative measurement of hematuria revealed significant elevation of hematuria score (p=0.0002), urine pus and protein (p=0.0005). Additionally, CBC-derived inflammatory biomarkers including neutrophil-lymphocyte ratio (NLR) (p=0.001), neutrophil-monocyte ratio (NMR) (p=0.0004), pro-inflammatory cytokine interleukin (IL)-6 (p=0.016) and tumor necrosis factor (TNF)-α (p<=0.007), total antioxidant capacity (TAC) (p<0.0001) were significantly increased. Evidence of obvious histopathological structural alteration was noticed in kidney tissue and bladder urothelium in CPH-treated animals. ZOF, TQ, and their co-treatment significantly prevented these deleterious effects associated with CPH treatment.

Conclusion: This study demonstrated that ZOF and TQ provided uroprotective and nephroprotective effects against CPH-induced nephrotoxicity by reducing kidney injury biomarkers, and CBC-derived inflammatory markers, restoring antioxidant capacity, and improving histopathological outcomes. The suggested mechanism involves the anti-inflammatory and antioxidant activity of TQ and the sulfhydryl-angiotensin converting enzyme inhibitor ZOF.

Keywords: anti-inflammatory, cyclophosphamide, hemorrhagic cystitis, kidney, oxidative stress

Background

Drug-induced nephrotoxicity is a major challenge in the treatment of various diseases. Approximately 20% of patients experience acute kidney injury following drug administration, with a significant number subsequently becoming susceptible to chronic kidney disease.[1,2] One of the significant concerns in cancer treatment is chemotherapy-induced nephrotoxicity and damage of the urinary tract that affects kidney function through different mechanisms. The prodrug

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cyclophosphamide (CPH) is a widely used anticancer drug, despite its crucial therapeutic effect, the toxicity of this drug is inevitable. Its toxicity is mainly related to the release of toxic metabolites "acrolein" by the liver and "chloroacetaldehyde" by the kidney, which has been extensively linked to CPH-induced hemorrhagic cystitis and renal dysfunction.

There are various mechanisms in CPH-induced urinary tract injury, mainly including the interaction of acrolein with the urothelium, the lining of the urinary tract, that enhances the inflammation via nuclear factor kappa B (NF-κB) up-regulation and increase expression and release of tumor necrosis factor-alpha (TNF-α), and interleukins (IL) like IL-1β and IL-6. Furthermore, oxidative stress has a crucial role in the induction of CPH-induced urinary tract injury.[3] Reactive oxygen species (ROS) generation mediated by CPH-metabolites reduces the anti-oxidant capacity and enhances lipid peroxidation in the renal system.[4–6]

Recent studies emphasize the significance of identifying kidney injury molecule-1 (KIM-1) and cystatin C, alongside markers such as neutrophil gelatinase-associated lipocalin (NGAL) for the early detection of acute kidney injury (AKI) and nephrotoxicity associated with CPH induced cystitis in clinical practice. These biomarkers exhibit superior sensitivity compared to traditional indicators. KIM-1 is effective in detecting proximal tubular damage, while cystatin C serves as a reliable marker for glomerular filtration rate assessment.[7,8]

Detoxification of CPH to reduce its urotoxicity can be achieved by vigorous diuresis and using sodium 2-mercapto ethane sulfonate (mesna).[9] However, mesna does not eradicate hemorrhagic cystitis symptoms and, at the same time, it is not practical in some cases as it was reported to cause hypersensitivity reactions ranging from mild dermatological effects to systemic anaphylactic reactions.[10]

Therefore, researchers are focusing on finding innovative therapeutic approaches, particularly by repurposing existing drugs, as an alternative strategy to mitigate the urotoxicity and nephrotoxicity caused by CPH. Zofenopril (ZOF) is a potent sulfhydrylated angiotensin-converting enzyme (ACE) inhibitor, it is a highly lipophilic molecule[11,12] that is known for its wide distribution in tissues, prolonged duration of action, and pleiotropic effects beyond lowering blood pressure.[13] It has notable antioxidant, and anti-inflammatory effects, increasing nitric oxide production and reducing ROS in endothelial cells.[12,14] In experimental models, ZOF protects against cerebral ischemia/reperfusion injury by mitigating oxidative stress and preventing apoptosis.[15] Various in vitro studies suggest that ZOF, due to the presence of a sulfhydryl group in its structure, releases hydrogen sulfide (H_2S),[13,16] and the presence of this unique sulfhydryl group in ZOF contributes to its superior effectiveness compared to non-sulfhydryl ACE inhibitors, particularly in improving vascular function and mitigating cardiac damage.[17,18] Additionally, ACE inhibitors have shown promise in the treatment of a variety of inflammatory conditions like pancreatitis, atherosclerosis, arthritis, and glomerulonephritis. Their anti-inflammatory effects contribute to renoprotective and cardioprotective properties, extending their therapeutic potential beyond the antihypertensive role.[19,20] These findings emphasize ZOF's diverse therapeutic potential, including antioxidant, anti-inflammatory, and antiapoptotic effects, alongside its primary function as an ACE inhibitor. Despite all these investigations, the therapeutic potential of sulfhydryl ACE inhibitors like ZOF has not yet been explored in the context of chemotherapy-induced nephrotoxicity and urotoxicity.

This study postulated that ZOF would be superior to other non-sulfhydryl ACE inhibitors in ameliorating the nephrotoxicity and urotoxicity associated with chemotherapeutic toxicants with less adverse effect, more affordability, and easy accessibility.

Additionally, many researchers highlighted the potential benefits of combining herbal medicines with conventional drugs to enhance efficacy and reduce toxicity. For instance, the use of melatonin, silibinin, and resveratrol as adjuvants with other medications has been tried in numerous preclinical and clinical studies,[21–23] including cases of drug-induced kidney injury, urotoxicity, and cardiotoxicity.

There is a growing body of research to show that many herbal medications with antioxidant and anti-inflammatory properties, such as thymoquinone, could ameliorate drug-induced nephrotoxicity, hemorrhagic cystitis, and cardiotoxicity.[24,25]

Thymoquinone (TQ); 2-Isopropyl-5-methyl-1, 4-benzoquinone is known for its powerful antioxidant, anti-inflammatory, and anti-angiogenesis.[26] Many experimental and clinical studies reported the importance of using TQ as a reno and uroprotective agent.[25,27] In an experimental in vivo study, TQ demonstrated protective effects against CPH-induced oxidative stress, as evidenced by a significant reduction in lipid peroxidation and the restoration of reduced glutathione levels, along with catalase and superoxide dismutase activities.[24] Furthermore, TQ treatment markedly inhibited DNA damage, as indicated by decreased DNA fragmentation, and enhanced Nrf2 expression in bladder tissues.[24] While several pharmacological interventions, including ZOF, have been shown to offer protection against various renal and cardiovascular diseases, their efficacy in preventing CPH-

induced nephrotoxicity is still not yet explored. Moreover, there are various encouragements for the adjuvant use of this substance with other drugs to attenuate chemotherapy-induced nephrotoxicity to improve renal function. Therefore, the current study was designed to investigate the uroprotective and renoprotective effect of ZOF and TQ individually or their co-treatment in CPH-induced hemorrhagic cystitis and nephrotoxicity in rats assessing these novel biomarkers.

Materials and Method

Chemicals and Reagents

Cyclophosphamide (Endoxan) and sodium 2-mercapto ethane sulfonate (Mesna) were obtained from Baxter Oncology GmbH, Frankfurt am Main, Germany. Zofenopril was obtained from A.Menarini Pharmaceutical Industry, Riunite S.r.I, Via Sette Santi, Florence, Italy. Thymoquinone was from Glentham Life Sciences (United Kingdom). Rat total antioxidant capacity (TAC), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), kidney injury molecule-1 (KIM-1), cystatin C (Cys-C) were measured by enzyme-linked immunosorbent assay (ELISA) kit from Bioassay technology laboratory, Shanghai, China. Kidney function biomarkers including serum creatinine, serum uric acid, blood urea, and serum total protein were measured using SIEMENS Dimension EXL 200 (Automated analyzer), serum electrolytes by ST- 200 Plus Electrolyte analyzer while complete blood counts (CBC) were measured by Mindray BC-5000 Automated Hematology Analyzer.

Urinalysis for hematuria was performed by using dipstick Urinalysis Reagent Test Strips manufactured by SureScreen Diagnostics Ireland Ltd-UK.

Animals and Ethical Statement

A total of 48 Wister Albino female rats of 8–10 weeks (weighing 170 ± 20g) were used for the study; they were obtained from the animal house of the College of Pharmacy-University of Sulaimani. The rats were kept in plastic cages and acclimatized for one week to a controlled environment, maintained at a temperature of 22 ± 1 °C, with 50± 5% relative humidity and a 12-hour-light-dark cycle. During the acclimatization and experimental period, each rats were kept in a single cage;[28] they had free access to the standard pellet diet and water throughout the study, except for the days when the rats were kept in individual metabolic cages, they had access only to water.

The protocol of this study was approved by the Ethical and Research Registration Committee of the College of Pharmacy-University of Sulaimani with a registration number (PH99-23 on May 24th, 2023). All procedures followed the standard principle of laboratory animal care and national institutional animal care.

Experimental Design and Treatment Protocol

The animals were divided into six groups of eight rats per each as follows:

1. The negative control (NC) group received 2 mL saline for 19 days, on the 17th day of the experiment a single injection of normal saline was given intraperitonially (IP).
2. The positive control (PC) group received saline for 19 days; on the 17th day of the experiment, a single dose of CPH 200 mg/kg body weight (BW) was given IP.
3. ZOF-15mg/kg group: 15mg/kg BW, ZOF was given orally for 19 days, and on the 17th day, a single dose of CPH 200 mg/kg BW was given IP.
4. TQ - 80mg/kg group: 80mg/kg BW, TQ was given orally for 19 days; on the 17th day, a single dose of CPH 200 mg/kg BW was given IP.
5. ZOF-15mg/kg with TQ 80mg/kg BW (TQ+ZOF) was given orally for 19 days, and on the 17th day, a single dose of CPH 200 mg/kg BW was given IP.
6. Mesna group; a total dose of 200 mg/kg BW, mesna in 20%, 40%, and 40% doses as a standard treatment for hemorrhagic cystitis was given IP 20 min before, 4 hours, and 8 hours after CPH injection, respectively, which was equal to 40 mg/kg, 80 mg/kg, and 80 mg/kg BW for each dose respectively.[29]

The dose and duration of CPH, ZOF, and TQ were selected depending on the previous studies,[26,30–32] with modifications.

Induction of Urothelial Injury and Nephrotoxicity

In each group except the negative control, urothelial injury, and nephrotoxicity were induced on the 17th day of the experiment. The rats were injected with CPH 200 mg/kg BW single dose in 2mL saline IP according to the methods described by Wrobel et al[33] and Rodo et al.[34] After 48 hours of CPH injection, the rats were sacrificed using chloroform inhalation. Following an abdominal incision, both kidneys and urinary bladders were removed and emptied of their urinary contents for future investigation.

Urine Collection

To collect urine samples, the metabolic cage was utilized. Each rat was placed in the metabolic cage for 24 hours. Urine was collected over 24 hours pre-CPH injection and 24 hours before the animal scarification,[35] its volume was measured, and urine analysis was performed by using urine dipstick analysis test strips. The urine samples were centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected. The samples were subsequently frozen at −80°C for future analysis.

Blood Collection

On day 19, following the animal's euthanization, approximately 6 mL of blood was collected from each rat via cardiac puncture. A portion of the blood was kept in an ethylenediaminetetraacetic acid (EDTA) tube for a CBC test, while the remaining blood was kept in a serum separator tube and centrifuged at 5000 rpm for 10 minutes at 4C° to collect the serum then the serum was frozen at −80°C for biochemical analysis.

Tissue Harvesting and Homogenization

The urinary bladder and both kidneys were removed by careful dissection. One kidney was homogenized, and the homogenate was used to determine the kidney-specific and other biochemical parameters. The other kidney and the whole urinary bladder were rinsed with ice-cold normal saline and placed in 25 mL of 10% formaldehyde for histopathological examination.

For the preparation of the tissue homogenate, the kidney was thoroughly rinsed with ice-cold phosphate buffer saline (PBS) to remove blood. It was weighed and then dissected into smaller pieces to facilitate homogenization and placed in a pre-chilled homogenizer tube with ice-cold PBS at a ratio of 9 mL per 1 gram of tissue. The homogenate was centrifuged at 5000 rpm for 5 minutes at 4C°; the supernatant was carefully collected without disturbing the precipitant, aliquoted into Eppendorf tubes, and stored at −80°C for kidney biomarker analysis.[36]

Measurement of Body Weight

The body weight of each rat was measured and recorded in the morning using standard weighing balance before starting the experiment, ie, before administration of each ZOF, TQ versus the last day of the experiment (48 hours post-CPH injection), and the change between the recorded data were calculated.

Assessment of Water Consumption

The water consumption was measured before and after CPH injection. To calculate the volume of water consumed by each rat, 100 mL water was supplied in a techniplast water bottle with a sipper cap containing a metal ball to control the access to water. Thus, the remaining volume of water in the bottle was subtracted from the initial 100 mL provided.

Parameters Determined in Urine

Urine Scan (Urinalysis)

Urine was collected over 24 hours before animal scarification for urine volume measurement, evaluation of hematuria, urine protein, and urine leukocytes.

Evaluation of Hematuria

Hematuria was evaluated in the urine samples collected in all groups 48 hours after CPH injection. The dipstick test[37] was used to assess the magnitude of hematuria semi-quantitatively from 0 to 4+ as follows: 0 = no hematuria, 1=Trace,

2=mild degree of hematuria, 3=moderate, 4= macroscopically detectable hematuria. This test has been used based on the previous study.[29]

Evaluation of Urine Urea, Creatinine, and Protein

The biochemical parameters assessed in the 24-hour urine sample after CPH administration were urine urea, creatinine, and protein using SIEMENS Dimension EXL 200 (Automated analyzer).

Parameters Determined in Blood and Tissue Homogenate

Conventional Kidney Function Biomarkers

A serum sample of each rat was used to determine the classical kidney function markers, including creatinine (μmol/L) (Cat. No. DF33B), uric acid (μmol/L) (Cat. No. DF77), BUN; urea nitrogen (mmol/L) (Cat. No. DF21), and total protein (g/L) (Cat. No. DF73) using the automatic analyzer SIEMENS Dimension EXL 200.

Kidney Specific Biomarkers

The concentration of cystatin C (Cys-C) ng/mL and kidney injury molecule-1 (KIM-1) ng/mL in the supernatant of kidney tissue homogenate were determined using Rat CYS-C (Cat.No.E0145Ra) and KIM-1 (Cat.No.E0549Ra) BT LAB ELISA Kit from Bioassay technology laboratory, Shanghai, China strictly following the manufacturer's instructions.

Pro-Inflammatory Cytokines

Kidney tissue homogenate was used to determine IL-6 (Cat.No. E0135Ra) and TNF-α (Cat.No.E0764Ra) using an ELISA kit (Bioassay technology laboratory, Shanghai, China).

Oxidative Status Parameters

Kidney tissue homogenate was used to determine total antioxidant capacity using a T-AOC (Cat.No. E3901Ra) ELISA kit (Bioassay Technology Laboratory, Shanghai, China).

Measurement of Complete Blood Count-Derived Inflammatory Markers

The calculation of CBC-derived inflammatory markers such as neutrophil-lymphocyte ratio (NLR), and neutrophil-monocyte ratio (NMR) was done using Mindray BC-5000 Automated Hematology Analyzer.

Histotechnique Procedure

The histological protocol was started at the experimental endpoint and the sections were stained with Harris's hematoxylin and eosin solution. Following the animal's euthanization, a necropsy was started to collect tissue samples for histological procedures. In brief, kidney, and bladder samples were immobilized and settled into tissue cassettes then fixed with 10% neutral buffered formaldehyde solution for about 48 hours. Thereafter, sections were dehydrated by passing through a series of ascending ethanol alcohol (50%, 60%, 70%, 90%, and 100%), followed by three steps of xylene clearance. Following that, the processed tissue samples were impregnated and embedded in melted paraffin blocks using an automated wax embedder at (60 −70^{o}C). Paraffinized tissues were sectioned to 5 μm using a semi-automated rotary microtome. After that, tissue sections were fixed on glass slides and dried using a hot plate tissue holder. Later, glass slides with their mounted tissue sections were deparaffinized and cleaned with xylene solution for 30 minutes then dried with a hot oven at 50^{o}C for 5 minutes. Lastly, glass slides with tissue sections were cleaned with xylene and cover slipped, then stained with Harris's hematoxylin and eosin solution and then viewed under a bright field light microscope.

Semi-Quantitative Lesion Scoring

Lesion scoring was estimated semi-quantitatively via image analyzer software (AmScope, 3.7) using a microscope eye-piece camera (MD500, 2019), and tissue samples were analyzed under the light microscope (NOVEL XSZ-N107T, China). Briefly, each kidney section fixed on the glass slides was divided into four subareas, then from each subdivision semi-quantitative evaluation of renal tubular vacuolar degeneration, cellular swelling was counted in each chosen four fields under high power magnification (100X), then the mean average was calculated statistically in percentage. Moreover, the area of hyaline cast deposition was measured in μm then later assessed in the mean percentage of the

given section. On the other hand, lesion scoring from sectioned bladder tissue is considered statistically based on the calculated mean percentage of acute cellular swelling, vacuolar degeneration, and epithelial hyperplasia within the selected sub-sections in addition to the mean percentage of areas of fibrous connective tissue proliferation in the lamina propria measured in µm.

Finally, the mean percentage of all calculated values were expressed as the following lesion grading scores (score 0–10% as no lesions; score 10–25% as mild; score 25–50% as moderate; score 50–75% as severe and score 75–100 as critical lesions).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 10.2.3. The Shapiro–Wilk test was used to examine the distribution of the variables. One-way ANOVA followed by Tukey's and Bonferroni tests was applied for group comparisons. Kruskal–Wallis test (One-way ANOVA) was performed for non-parametric data adjusted by Dunn'post-hoc test to indicate significant differences between different groups. A p-value of less than 0.05 was considered significant.

Results

Effects of ZOF, TQ, and Their Combination on the Rat's Body Weight

The changes in the body weight of all animals are shown in Figure 1. There was a significant reduction in the body weight of the PC group when compared to the NC group (p=0.0009). In ZOF treated group, a significant increase in the rat's body weight (p=0.0066) versus PC was observed. Additionally, in the other groups, the rat's body weight was increased in a non-significant manner (p>0.05).

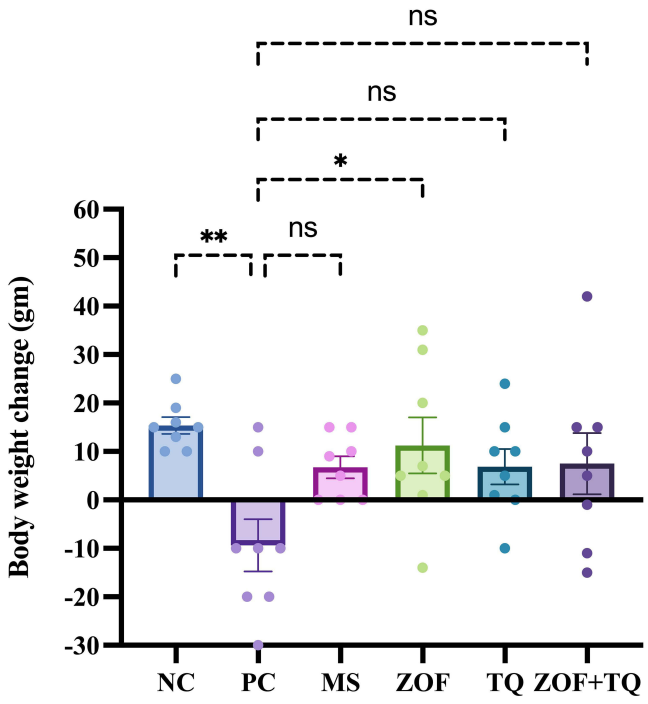

Figure 1 Changes in Rat's Body Weight Before Starting the Experiment versus the Last Day of the Experiment. One-way ANOVA multiple comparison followed by Tukey's test was used, number of animals per each group=8. *p =0.0286, **p=0.005.
Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; ns, non-significant.

Water Consumption and Urine Volume Assessment

Effects of ZOF, TQ, and Their Combination on Water Consumption and Urine Volume

In the present study, water consumption was increased in the ZOF group before and after CPH administration, showing a significant increase in comparison to the NC (p=0.0009, p=0.0155 before and after CPH respectively) and PC groups (p=0.0009 before and after CPH). Meanwhile, water consumption in the ZOF+TQ group was non-significantly higher than in the NC and PC groups, but it significantly decreased (p=0.0185) following CPH administration (Table 1).

Urine volume was decreased after CPH administration in both PC and MS in a non-significant manner (Table 2). Additionally, urine volume was increased in the ZOF group before and after CPH administration, showing a significant increase in comparison to the NC (p=0.0002, p=0.002 pre and post-CPH respectively) and PC (p=0.0001) groups. Furthermore, in the ZOF+TQ group urine volume was non-significantly higher than NC and PC groups, but it significantly decreased (p=0022) following CPH administration.

Table 1 Water Consumption Over 24 hours Before and After CPH Administration (n=8)

Water Consumption (mL)	NC	PC	MS	ZOF	TQ	ZOF+TQ
Pre-CPH (Mean ±SD)	22.94±6.27	16.50±4.38	16.13±6.73	44.0±9.68$^{***\#\#\#\#\#}$	26.88±12.36	30.63±10.78^{a}
Post-CPH (Mean± SD)	24±6.63	16.4±8.91	10.8±4.33	40.8±22.1$^{*\#\#\#\#\#}$	23±9.47	19.8±4.98^{b}

Notes: Values are presented as mean±SD. Two-way ANOVA multiple comparison was used and confirmed by Tukey's test. n: number of rats per each group. Significant differences are indicated by *p=0.0155, ***p=0.0009 vs control. $^{\#\#\#\#\#}$p=0.0001 vs PC. Non-identical superscript letters (a,b) indicate significant differences between the same group at different times.
Abbreviations: NC, negative control; PC, positive control; MS, mesna group; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide; SD, standard deviation.

Table 2 Urine Volume Collected Over 24 hours Before and After CPH Administration (n=8)

Urine Volume (mL)	NC	PC	MS	ZOF	TQ	ZOF+TQ
Pre-CPH (Mean ±SD)	13.9±1.5	11.8±1.5	14.9±2.7	38.7±12.1$^{***,\#\#\#\#\#}$	14.3±5.0	24.8±8.2^{a}
Post-CPH (Mean± SD)	13.6±1.7	6.3±3.4	6.8±2.0	35.1±19.6$^{***,\#\#\#\#\#}$	11.4±9.6	12.3±7^{b}

Notes: Values are presented as mean±SD. Two-way ANOVA multiple comparison was used and confirmed by Tukey's test. n: number of rats per each group. Significant differences are indicated by ***p=0.0002 vs NC $^{\#\#\#\#\#}$p=0.0001 vs PC. Non-identical superscript letters ^{a,b}indicate significant differences between the same group at different times.
Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide; SD, standard deviation.

Impacts of ZOF, TQ, and Their Combination on Urinalysis Parameters by Dipstick Test

The effects of ZOF, TQ, and ZOF+TQ on urinalysis parameters are shown in Figure 2A–C. Figure 2A depicts the semiquantitative assessment of hematuria on dipstick tests. The result revealed that the PC group showed a significantly higher (P=0.0002) hematuria score than the NC group. Analysis of the urine pus (leukocytes) and proteins also demonstrated a significantly higher level in the PC group versus NC group (p=0.0005 and p=0.0007 respectively). Additionally, these parameters demonstrated higher score in the PC group in comparison to other treated groups; ZOF, TQ, and ZOF+TQ as shown in Figure 2B and C. However, in ZOF, TQ, and their combination there were a significant reduction of these urinalysis parameters parallel to the MS group.

The representative image results of dipstick tests for proteinurea and hematuria are shown in Figure 3A and B respectively.

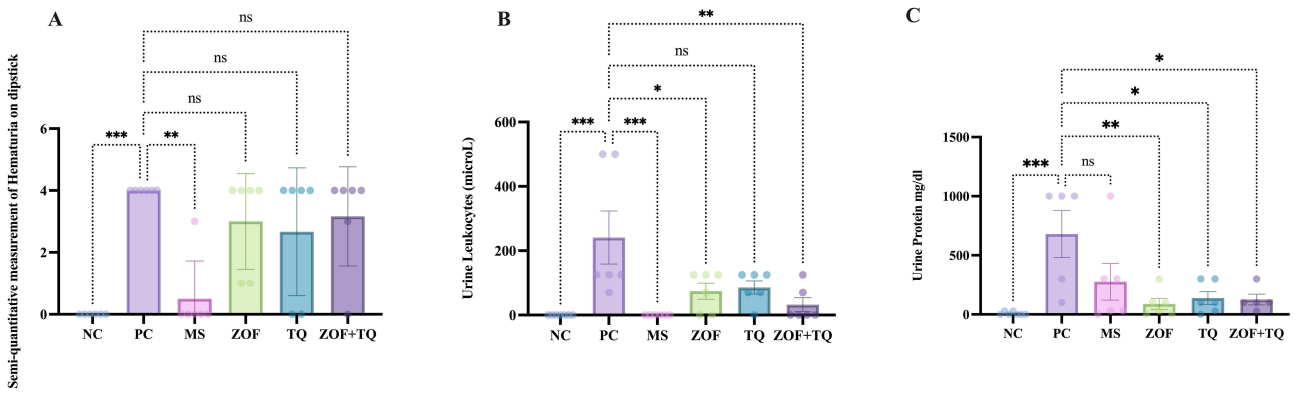

Figure 2 Effect of ZOF, TQ, and their combination on urinalysis parameters by dipstick test 48 hours following CPH injection. (**A**) Hematuria measured semi-quantitatively, (**B**) Urine Leukocytes, (**C**) Urine Protein. The Kruskal–Wallis test was performed for non-parametric data followed by Dunn'post-hoc test for hematuria analysis. While one-way ANOVA was used for urine pus and protein. Number of animals per each group=8. Data are presented as means ±SEM. **p =0.001, ***p = 0.0002 in 2A. *p=0.325, **p=0.0042, ***p=0.0005 in 2B. *p=0.01, **p=0.0045, ***p=0.0007 in 2C versus PC group are significantly different.
Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide; SEM, standard error of the mean.

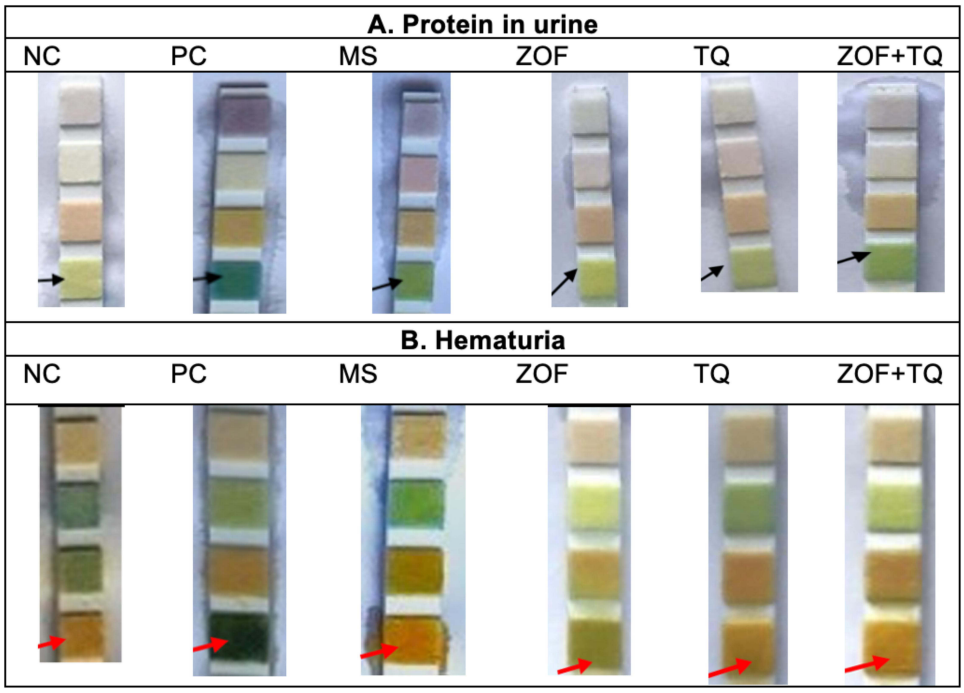

Figure 3 Representative images of dipstick tests for (**A**) Protein in urine and (**B**) Hematuria.Black arrows show the area of proteinuria detection in the dipstick test. Red arrows show the area of hematuria in the dipstick test.
Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide.

Effects of ZOF, TQ, and Their Combination on Biochemical Parameters in Urine

The biochemical parameters measured in the urine are shown in Table 3. CPH disturbed the urinary excretion of urea, creatinine, and protein. However, ZOF slightly preserved the kidney function by reducing their excretion non-significantly. TQ alone and in combination with ZOF has no significant effect on these variables after CPH injection.

Table 3 Biochemical Parameters Assessed in Urine Collected Over 24 hours After CPH-Administration (n=8)

Urine Parameters-After CPH Administration (Mean ±SD)	NC	PC	MS	ZOF	TQ	ZOF+TQ
Urine Urea mmol/L	257.1±27.9	584.1±105	698.1±126.9	358.5±67.6	523.2±79.8	628.5±95.6
Urine Creatinine micro mol/L	3884.4±714.3	4448.9±797	6764.38±1003.3	2440.25±592.9	4421±631.3	5376.25±1694.5
Urine Protein mg/dl	29.9±7.8*	227.8±65.97	53.3±10	95.26±38.8	196.8±47.6	133.4±42.2

Notes: Values are presented as mean±SD. One-way ANOVA multiple comparison was used and confirmed by Tukey's test. n: number of rats per each group. *Significant difference is indicated by *P=0.025 vs PC.

Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide; SD, standard deviation.

Effects of ZOF, TQ, and Their Combination on Conventional Kidney Function Biomarkers Assessed in Serum

As shown in Figure 4A–D, CPH resulted in a non-significant alteration in the serum level of conventional kidney function biomarkers; creatinine, uric acid, and blood urea, except total protein which was significantly reduced in rats injected with CPH). Serum uric acid was reduced in the groups treated with ZOF, TQ, and their combinations, while blood urea level was elevated.

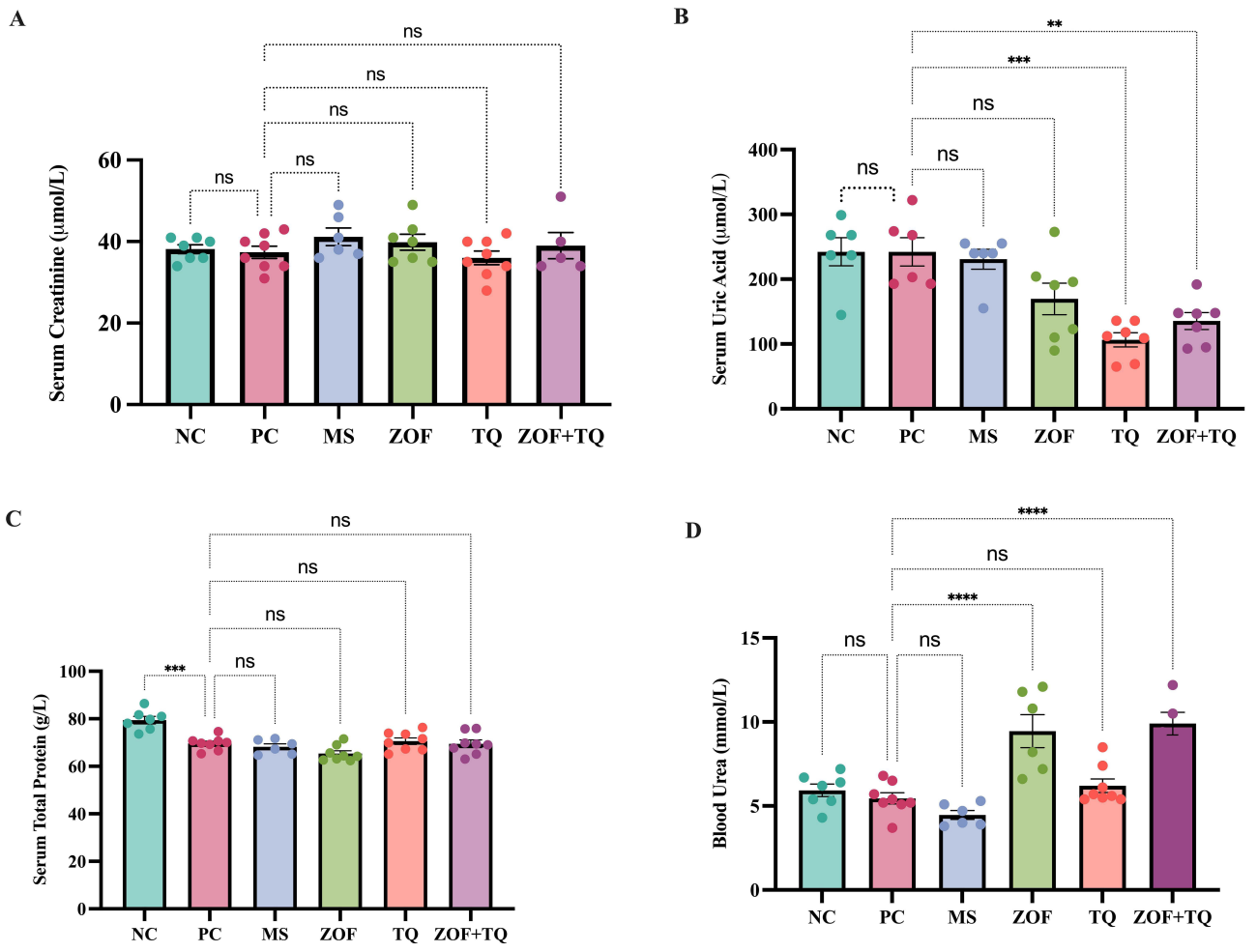

Figure 4 Effect of ZOF and TQ alone and their Combinations on Conventional Kidney Function Parameters. (**A**) Serum Creatinine, (**B**) Serum Uric Acid, (**C**) Serum Total Protein, (**D**) Blood Urea. One-way ANOVA multiple comparison followed by Tukey's test was used, number of animals per each group=8 ns: indicates statistically non-significant in 4A. **p=0.0034, ***p=0.0001 in 4B, ***p=0.0001 in 4C, ****p<0.0001 in 4D are statistically significant compared with PC group.

Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide.

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Effects of ZOF, TQ, and Their Combination on Novel Biomarkers for Kidney Injury in Tissue Homogenate

In the present study, the level of new protein biomarkers KIM-1 and Cys-C was evaluated in kidney tissue homogenate following CPH-injection. The level of KIM-1 was non-significantly elevated (p=0.27), while Cys-C was elevated significantly (p=0.019) in tissue homogenate following CPH-injection (Figure 5A and B). KIM-1 and Cys-C levels were significantly decreased in the groups treated with MS, ZOF, TQ, and the ZOF and TQ combination (p=0175, p=0.007, p=0.04 and p=0.005 respectively in KIM-1) and (p=0.007, p=0.0343, p=0.007 and p=0.0319 respectively in Cys-C).

Effect of ZOF, TQ, and Their Combination on Complete Blood Count-Derived Inflammatory Markers

To investigate the role of inflammation in kidney and urinary bladder injury, the impact of CPH on blood cell components was analyzed. Complete blood count-derived inflammatory biomarkers including NLR and NMR were measured. As displayed in Figure 6A and B, induction with CPH resulted in a significant increase in the level of NLR and NMR (p=0.001 and p=0.0004 respectively). However, treatment with ZOF, TQ, and their combination ameliorated NLR in a non-significant manner (p=0.999, p=0.361, and p=0.999 respectively), and significantly reduced NMR (p=0.04, p=0.01, p=0.0008 respectively).

Effects of ZOF, TQ, and Their Combination on Pro-Inflammatory and Oxidative Stress Markers

The effects of ZOF, TQ, and their combination on the inflammatory biomarkers were investigated. Figure 7A and B showed a significant increase in the pro-inflammatory cytokine IL-6 and TNF-α levels in the kidney tissue homogenates following CPH injection (p=0.0165, p=0.0074 respectively). However, these deleterious changes in IL-6 caused by CPH were attenuated by ZOF in a non-significant manner (p=0.81), while significantly by TQ, and ZOF+TQ combinations (p=0.0001 in both). Additionally, oxidative stress was significantly elevated after CPH injection represented by TAC level in Figure 7C. ZOF and TQ showed a non-significant restoration of the antioxidant capacity of the animals (p=0.3819 and p=0.897 respectively). Meanwhile, their combinations significantly elevated TAC levels in kidney tissue homogenate (p=0.012).

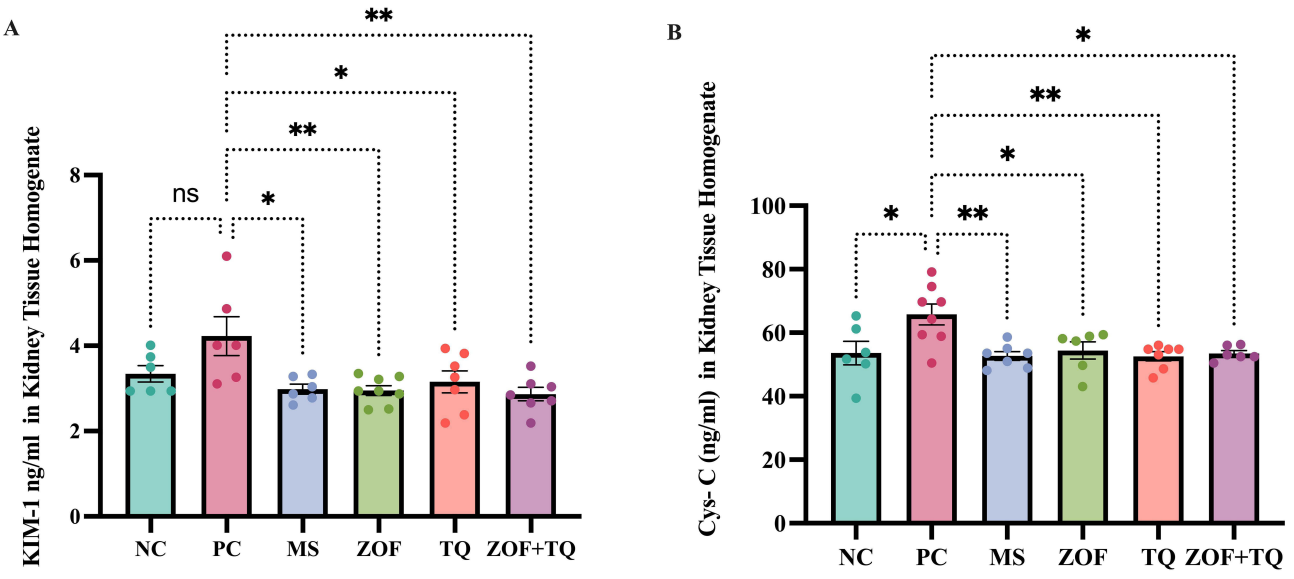

Figure 5 Effect of ZOF and TQ alone and their combination on Kidney Injury Biomarkers. (**A**) KIM-1 and (**B**) Cys-C in Kidney Tissue Homogenate. One-way ANOVA multiple comparison was used followed by Tukey's test. Number of animals per each group=8. *p=0.0175 PC vs MS, **p=0.007 PC vs ZOF, *p=0.04 PC vs TQ, **p=0.0055 PC vs ZOF+TQ in 5A; *p=0.019 PC vs NC, *p=0.031 PC vs ZOF+TQ, **p=0.007 PC vs MS and TQ, in 5B are statistically significant.

Abbreviations: Cys-C, cystatin C; KIM-1, kidney injury molecule-1; NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide; SD, standard error of the mean (SEM).

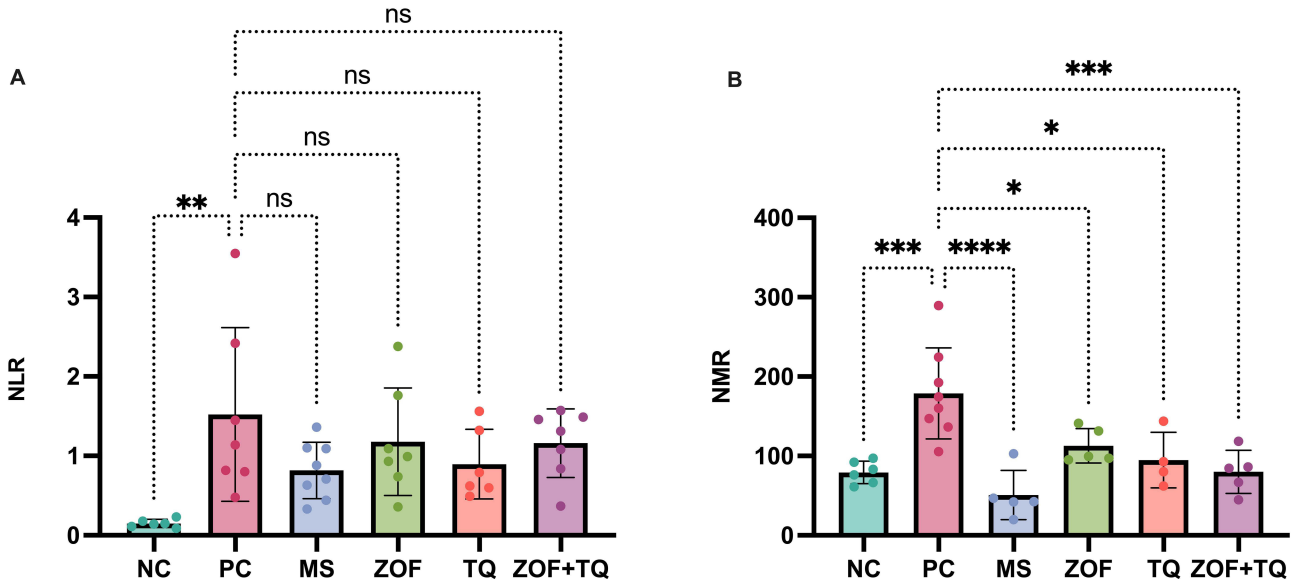

Figure 6 Effect of ZOF, TQ, and their combinations on Complete Blood Count-Derived Inflammatory Markers. NLR (**A**), NMR (**B**). One-way ANOVA multiple comparison was used followed by the Bonferroni test. Number of animals per each group=8.In 6A**p= 0.001 PC vs NC, in 6B *p=0.04 PC vs ZOF, *p=0.01 PC vs TQ, ***p=0.0004 PC vs NC, ***p=0.0008 PC vs ZOF+TQ, ****p=0.0001 PC vs MS are statistically significant.
Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide.

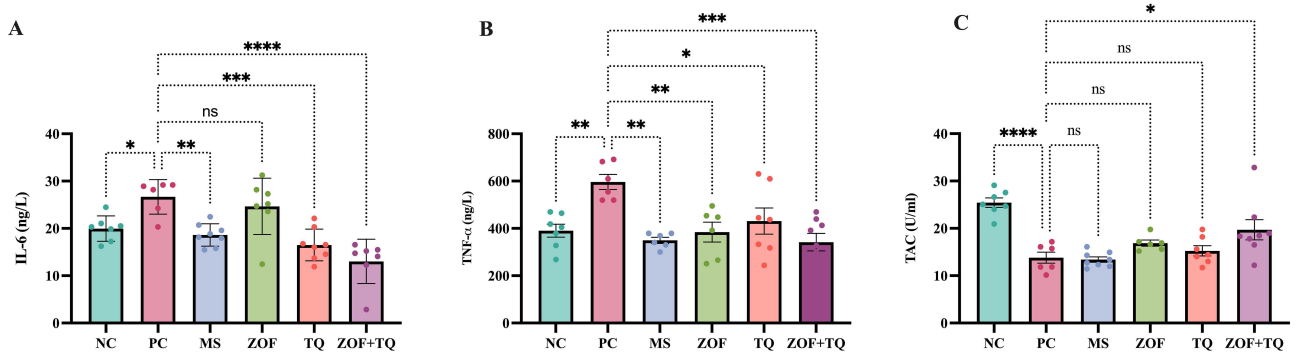

Figure 7 Effects of ZOF, TQ, and their combinations on Pro-inflammatory Cytokines and Total Antioxidant Capacity. IL-6 (**A**), TNF-α (**B**), TAC (**C**). Data was analyzed by One-way ANOVA multiple comparison followed by Dunnett's test. Number of animals per each group=8. *p<0.05, **p<0.007, ***p<0.0007, ****p<0.0001 are statistically significant compared with PC group.
Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone; CPH, cyclophosphamide.

Histopathology Findings

Kidney

As an overall concept, Table 4 establishes the semi-quantitative morphometric analysis and lesion scoring of kidney sections. At first, renal histological sections from the PC group demonstrate severe and significant tubular epithelial vacuolar degeneration and acute cellular swelling, in addition to profound glomerular atrophy together with the critical grade of protein accumulation within the renal tubular lumina which refers to hyaline cast, in comparison to sections from the NC group, which represent normal and typical morphology of kidney tissue. However, remarkably treatment with both ZOF and TQ as a regimen dose of 15mg/kg BW and 80mg/kg BW respectively, as a separated and combination remedy revealed a significant P<0.05 reduction in the percentage of cellular swelling within the renal tubular epithelia as well as an overall lesion severity mitigation. The prophylactic effect of the therapy was much more significant and effective in the combination group (ZOF&TQ) which reduced the lesion scoring profoundly from critical and severe to moderate grade. Moreover, tissue sections from animals that received mesna protocol, still display significant lesion

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Table 4 Semi-Quantitative Evaluation of Kidney's Histological Sections (n=8)

Experimental Groups n=8	Vacuolar Degeneration* (Mean %)**	Cellular Swelling* (Mean %)**	Hyaline Cast* (Mean %)**	Lesion Scoring (0 −100%)	Lesion Grading
NC	6.32%^{A}	7.18%^{A}	2.87%^{A}	0–10%	No lesion
PC	89.35%^{E}	91.62%^{E}	82.73%^{E}	75–100%	Critical
MS	63.44%^{D}	67.58%^{D}	58.42%^{D}	50–75%	Severe
ZOF	37.26%^{C}	45.31%^{C}	34.86%^{C}	25–50%	Moderate
TQ	60.54%^{D}	63.56%^{D}	53.39%^{D}	50–75%	Severe
ZOF+TQ	29.47%^{C}	36.97%^{C}	25.47%^{C}	25–50%	Moderate

Notes: *Renal tubules vacuolar degeneration and acute cellular swelling were estimated in mean (%) of counted cell numbers. Areas of hyaline casts were estimated in mean percentage of (μm). **Each value represents a mean percentage, the number of animals per each group=8. Mean values with different capital letters (A,C,D and E) have significant differences at ($P < 0.05$).
Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone.

scoring represented by severe lesion grade; however, it was much lesser than the PC group evident by critical grade, and more than 75% in lesion scoring evaluation. Additionally, Figure 8 represents the histopathological comparison among the treated groups which reveals significant improvement in the epithelial foundations in comparison to the PC group.

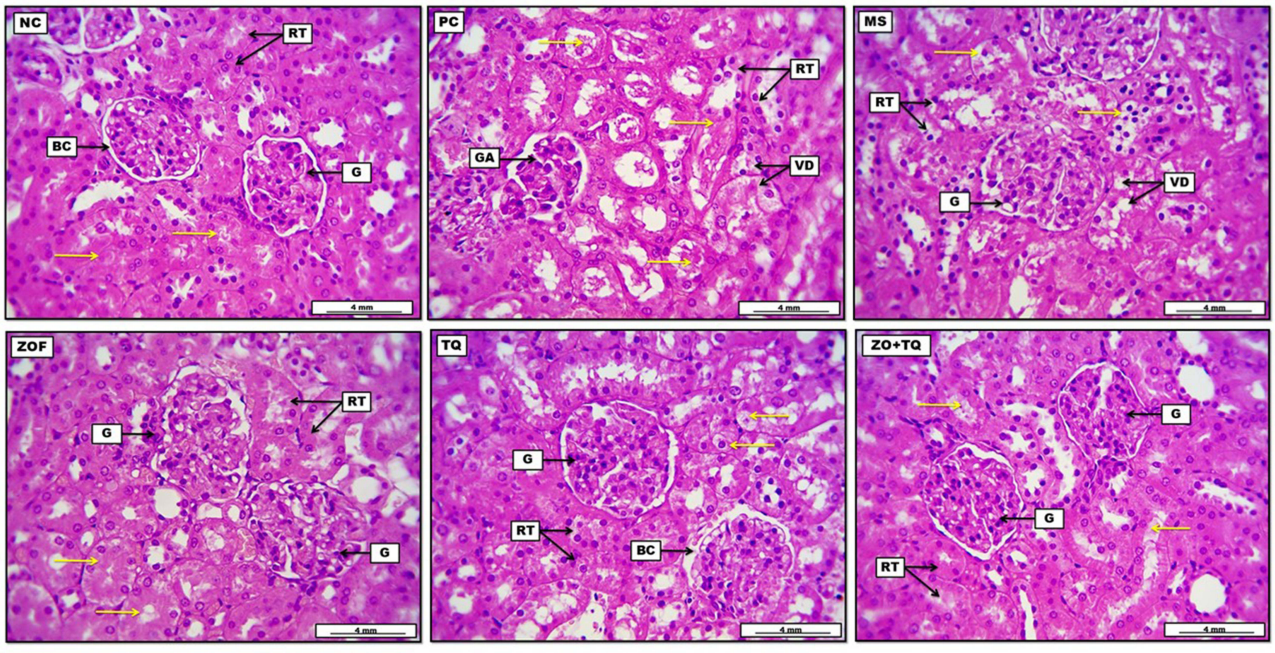

Figure 8 Photomicrograph of the kidney from groups; NC: negative control group, displays no obvious morphological changes except for slight and nonsignificant tubular cellular swelling (yellow arrows), presence of typical glomeruli (G) with standard Bowman's capsule (BC). Renal tubules (RT) exhibit no significant and serious pathological lesions in the given section. PC: positive control group which reveals significant glomerular atrophy (GA), moreover the renal tubular epithelia show severe vacuolar degeneration (VD) and cellular swelling, together with the accumulation of pinkish proteinaceous hyalinous casts within their lumina (yellow arrows). MS: mesna group, shows significant vacuolar degeneration (VD) and cellular swelling (yellow arrows) within the renal tubular epithelium (RT) within the given section. On the other hand, glomerular structure (G) reveals no evidence of morphological alteration. ZOF: Zofenopril group, demonstrates moderate cellular swelling (yellow arrows) in the renal tubular epithelia (RT) evident with narrowing of their lumina. Moreover, other areas of the given section, show normally appeared renal tubules (RT) as well as some regular glomeruli (G). TQ: Thymoquinone group reveals a normally appeared glomerular structure (G) with slight congestion in the Bowman's capsule (BC), moreover, renal tubular epithelia (RT) display significant cellular swelling apparent by its turbid and granular cytoplasm (yellow arrows). ZO+TQ: zofenopril and thymoquinone group shows typically appeared renal tubules (RT) with moderate cellular swelling and slight luminal accumulation of pretentious casts (yellow arrows), glomerular structures (G) appear intact with no indication of abnormal morphological deviations. H&E. Scale bar: 4 mm.

Urinary Bladder

In general, according to the semiquantitative morphometric evaluation demonstrated in Table 5, the bladder sections in both control positive and mesna protocols groups, show the significant and diffuse distribution of degenerative cells within the superficial epithelial layer represented by distinct acute cellular swelling, epithelial hyperplasia, and multifocal mucosal sloughing, explained as a critical lesion in PC group and alleviated severe lesion in mesna group. On the other hand, these lesion-scoring indicators have been improved significantly in the medicated groups and reduced clearly to a moderate lesion score. Still, a great reduction in the percentage of degenerative epithelial cells was seen in the combination group of ZOF and TQ in comparison to the ZOF and TQ alone groups, which shows a significant dropping in lesion severity to below 40%. Furthermore, Figure 9 represents the histopathological comparison among the treated groups which reveals significant improvement in the epithelial foundations in comparison to ruined ones.

Discussion

Cyclophosphamide is one of the widely used chemotherapeutic agents for the treatment of a broad spectrum of malignancies. Numerous studies have shown that the urotoxicity and nephrotoxicity of CPH's metabolites; acrolein and chloroacetaldehyde, are serious limitations in cancer therapy.[4,38] In the present study, CPH as a single IP injection of 200 mg/kg has been used for urothelium and kidney injury as previously described by other studies.[5,28,29,39] This model provides multiple mechanisms for urinary tract injury. The CPH's metabolites on the one hand and the generation of the reactive free radicals and production of numerous inflammatory mediators on another hand, play a crucial role in the molecular mechanisms of acrolein-induced hemorrhagic cystitis and the toxic sequala of the CPH.[24]

Inflammation plays a crucial role in the development of AKI, various models, including ischemia, sepsis, and nephrotoxicity, suggest that the initial damage to the kidney results in functional and/or structural alterations in the tubular epithelium or vascular endothelium. Subsequently, leukocytes including lymphocytes, neutrophils, natural killer cells, and macrophages are infiltrated into the injured kidneys. The injury prompts the release of inflammatory mediators like chemokines and cytokines from endothelial and tubular cells, which further attract leukocytes into the kidney.[40] Therefore, the treatment of CPH-induced urinary tract toxicity is complex, the current researches focus on targeting these mechanisms with inhibitors.[4,41]

While the development of new natural or synthetic agents requires extensive preclinical and clinical testing, repurposing already approved drugs with established safety and pharmacokinetic profiles offers a promising strategy to accelerate the introduction of effective treatments. Many reports suggested that ACE inhibitors particularly the sulfhydrylated types are convenient to be repurposed as adjuvant to the standard chemotherapy as oncolytic and oncopreventive[42] and other evidence showed that ACE inhibitors may have applications in the clinical setting beyond its antihypertensive effect.[43] Zofenopril, a sulfhydrylated type of ACE inhibitor possessing remarkable antioxidant and anti-inflammatory properties, protects tissues against various noxious stimuli.[12,13] In this study, the protective effect of

Table 5 Semi-Quantitative Evaluation of Urinary Bladder Histological Sections (n=8)

Experimental Groups n=8	Cellular Swelling* (Mean %)**	Epithelial Hyperplasia* (Mean %)**	Tissue Proliferation* (Mean %)**	Lesion Scoring (0 −100%)	Lesion Grading
NC	3.24%^{A}	2.58%^{A}	8.36%^{A}	0–10%	No lesion
PC	89.26%^{E}	86.19%^{E}	88.53%^{E}	75–100%	Critical
MS	73.32%^{D}	71.38%^{D}	69.49%^{D}	50–75%	Severe
ZOF	44.18%^{C}	42.79%^{C}	39.82%^{C}	25–50%	Moderate
TQ	42.75%^{C}	36.55%^{C}	45.19%^{C}	25–50%	Moderate
ZOF+TQ	32.21%^{C}	28.76%^{C}	38.49%^{C}	25–50%	Moderate

Notes: *Acute cellular swelling and epithelial hyperplasia within the lining mucosa were estimated in the mean (%) of counted cell numbers. Areas of connective tissue proliferation in the lamina propria were estimated in mean percentage of (μm). **Each value represents a mean percentage (n=8). Mean values with different capital letters (A,C,D and E) have significant differences at ($P < 0.05$).

Abbreviations: NC, negative control; PC, positive control; MS, mesna; ZOF, zofenopril; TQ, thymoquinone.

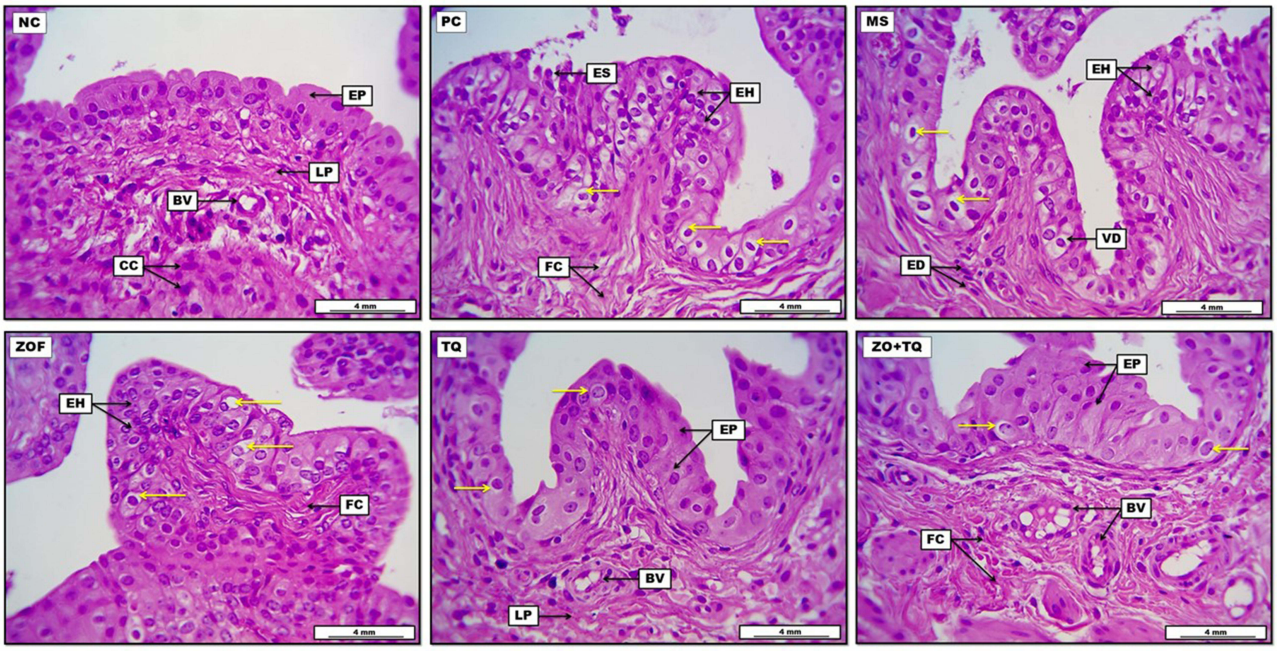

Figure 9 Photomicrograph of Urinary Bladder from groups; NC: negative control group, reveals no obvious pathological changes, evident by distinctive transitional epithelium (EP), with typical lamina propria (LP) consisting of loose connective tissue which contains cross-sectional blood vessels (BV), and normally distributed connective tissue cells (CC). PC: In the positive control group, the area of the mucosa demonstrates significant epithelial hyperplasia (EH), together with a multifocal area of epithelial sloughing (ES). The section also reveals significant acute cellular swelling within the upper mucosal layer (yellow arrows), and the lamina propria preview distinctive fibrous connective tissue proliferation. MS: mesna group, shows significant vascular degeneration and acute cellular swelling (yellow arrows) among the mucosal layer which also demonstrates some degree of epithelial hyperplasia (EH), in addition, there is a pinkish area of edematous cellular exudates within the submucosal proliferated connective tissue (ED). ZOF: The zofenopril group expresses the presence of significant epithelial hyperplasia (EH) together with the presence of low grade acute cellular swelling (yellow arrows) within the proliferated epithelia, in addition to the presence of some mild to moderate fibrous proliferation (FC). TQ: thymoquinone group displays no significant morphological disruption, only for a moderate degree of cellular swelling (yellow arrows) evident by typically settled transitional epithelial surface (EP), together with naturally appeared lamina propria with some blood vessels (BV) in the given section. ZO+TQ: zofenopril and thymoquinone group demonstrate non-significant cellular swelling (yellow arrows) with ordinary arranged transitional epithelial tissue (EP), Additionally, there is a moderate fibrous connective tissue proliferation within the submucosal layer (FC) together with non-significant vascular congestion (BV). H&E. Scale bar: 4 mm.

ZOF, TQ, and their combination against CPH-induced hemorrhagic cystitis was investigated. For the first time, ZOF individually and in combination with TQ exhibited reno and bladder urothelium protective effects through its potential antioxidant and anti-inflammatory effects and the biochemical restoration in blood and kidney tissue homogenate.

Recent studies have focused on the predictive value of the inflammatory markers derived from CBC in various clinical settings including kidney impairments and cancer, and measurement of NLR, NMR, and PLR were provided significant prognostic tools in early diagnosis and management of complications.[44,45] Furthermore, evaluation of CBC-derived inflammatory markers including NLR and PLR has been performed in clinical settings for patients with metastatic renal cell cancer treated with tyrosine kinase inhibitors.[46]

In the current study, NLR and NMR were significantly increased after CPH injection, and treatment with ZOF, and TQ, and their combination significantly reduced their value which indicates the anti-inflammatory effects of these tested agents in this model. The anti-inflammatory effect of ZOF is attributed to the ability of ACE inhibitors in reducing neutrophil counts, consequently, reducing NLR which serves as a reliable marker for inflammation. This finding aligns with a recent study that demonstrates the anti-inflammatory effect of ACE inhibitors through a significant NLR reduction in patients with heart failure.[47] Many studies highlighted the anti-inflammatory effect of TQ, however, to the best of our knowledge none of them targeted CBC-derived inflammatory biomarkers such as NLR and MNR. Therefore, these agents appear promising in protecting against kidney injury by various insults. Although the significance of NLR, PLR, and MLR in patients with certain hematologic malignancies has been previously investigated,[48] our study was the first to examine various CBC-derived biomarkers during interventions against chemotherapy-induced nephrotoxicity and urotoxicity.

In this experimental model, the laboratory investigations, macroscopical results with dipstick tests, histopathological analyses, oxidative stress, and inflammatory biomarkers parallel to the other studies[33] demonstrated the onset of kidney injury secondary to CPH-induced hemorrhagic cystitis and nephrotoxicity. For renal function assessment, this study applied both traditional nitrogenous serum markers and novel renal biomarkers such as Cys-C and KIM-1 in tissue homogenates in addition to CBC-derived inflammatory biomarkers to evaluate kidney injury. Due to their advanced sensitivity and specificity, modern nephrology diagnostics, favor the emerging novel biomarkers over the conventional nitrogen parameters.[49]

In the assessment of the living parameters, CPH resulted in a significant reduction in the body weight of the animals, similar finding has been demonstrated in another study conducted by Merwid-Lad et al.[29] On the contrary, another study has shown an increased bladder index without a reduction in the total body weight of rats following a 200 mg/kg dose of CPH.[50] These discrepancies may be related to different scarification timelines of the rats as the present study sacrificed the animals after 48 hours, while the other study after 24 hours.[50] Body weight was significantly increased in the ZOF-treated group while non-significant changes have been observed in TQ and its combination with ZOF groups. This might be attributed to the TQ's weight reduction impact on the rats.[51] The animal's water consumption over 24 hours in the metabolic cage was also assessed, ZOF significantly increased water intake and urine output, and it induced polydipsia and polyuria. This effect is consistent with the dispogenic effect of captopril which was elucidated by bradykinin's stimulation of thirst.[52] In the present study, the increase in water consumption (polydipsia) in the ZOF-treated group followed by increased urine volume (polyuria), might add another protection value that cleans up the renal tubules against urotoxic and nephrotoxic CPH's metabolites.

In the present study, slight changes in the conventional renal function biomarkers including serum creatinine, uric acid, and blood urea have been observed after CPH injection, except serum total protein which was significantly decreased following CPH induction. The non-significant alteration in these conventional biomarkers in the present study might be attributed to the unreliability of these biomarkers in acute kidney injury as serum creatinine level can take several days to reach a new steady state and does not reflect the true decrease in glomerular filtration rate, thus, conventional biomarkers lack sensitivity for early detection and diagnosis of kidney injury. [53], [54] The use of ZOF was able to decrease the serum level of uric acid non-significantly, meanwhile, TQ and its combination with ZOF significantly decreased it. The nephroprotective effect of ACE inhibitors was reported in various pre-clinical and clinical studies and amelioration of these traditional biomarkers has been reported remarkably in many studies. [55–57] The nephroprotective effect of ZOF has been documented further in several experimental models of kidney damage including renal ischemia-reperfusion injury[58] and diabetes-induced nephropathy[57] in which ZOF was able to reduce the serum level of creatinine, urine protein, and oxidative stress.

Furthermore, a recent study has demonstrated the renopreventive effect of TQ in chemotherapy-induced nephrotoxicity via amelioration of these biomarkers.[59] Many studies reported improvement in conventional renal function biomarkers and glomerular filtration rate, as shown in vancomycin-induced nephrotoxicity, TQ exerted a protective mechanism via attenuation of oxidative stress and decrease in serum creatinine and blood urea nitrogen,[60] similar results were obtained in the context of conventional markers in amikacin-induced renal injury in rats.[61]

The efficacy of ZOF and TQ has also been assessed by using novel biomarkers for acute kidney injury including KIM-1 and Cys-C. Although the value of KIM-1 and Cys-C in chemotherapy-induced AKI has been studied previously both in vitro and in vivo,[62] to the best of our knowledge, the present study is the first of its kind to investigate the efficacy of ZOF using these novel biomarkers in chemotherapy-induced urotoxicity and nephrotoxicity and it has been found that ZOF and TQ were significantly decreased the level of these biomarkers in kidney tissue homogenate.

Additionally, amelioration of pro-inflammatory biomarkers (IL-6 and TNF-α) has been observed markedly in the current study. In ZOF and TQ groups adding TQ to ZOF potentiates ZOF's action and synergizes its anti-inflammatory effects. Thus, these substances have a potential protective effect against chemotherapy-induced renal injury in multifaced mechanisms.

The finding of the current study is consistent with the results of captopril, an SH-containing ACE inhibitor, that attenuated cardiac and renal inflammation in a model of spontaneously hypertensive rats by reducing pro-inflammatory cytokines IL-1B, and IL-6, and increasing anti-inflammatory cytokines including IL-10. These effects were achieved through the inactivation and amelioration of NF-κB signaling pathway.[19,20]

Moreover, in another study, TQ exhibited a protective effect against CPH-induced hemorrhagic cystitis via decreasing the level of pro-inflammatory cytokines including IL-6 and TNF-α and restoration of the endogenous antioxidant mechanisms, amelioration of lipid peroxide metabolism and protecting the urinary bladder through Nrf2 signaling pathway,[24] which is consistent with the results obtained from the current study. Various studies showed that TQ attenuated kidney damage induced by several chemotherapeutic drugs.[63] The nephroprotective effect of TQ was related to enhancing the expression of Nrf2 and HO-1, inhibiting NF-κB,[64] reducing lipid peroxidation, improving antioxidant enzyme levels, and suppressing the inflammatory markers.[65]

The antioxidant ability of ZOF and TQ has also been investigated in this study, it has been found that CPH disturbed the total antioxidant capacity in the animals, meanwhile, ZOF and TQ increased TAC non-significantly, and their combination restored TAC in a significant manner, this indicates the synergistic effect of TQ when combined with ZOF. The antioxidant and renoprotective effects of ZOF have been demonstrated in many preclinical studies as shown in N-nitro-L-Arginine Methyl Ester (L-NAME) induced hypertension, ZOF ameliorated oxidative stress biomarkers along with activation of the antioxidant defense system.[66] The sulfhydryl-containing ACE inhibitor ZOF is a source of hydrogen sulfide, therefore, due to the release of this molecule, ZOF exerts an extra beneficial effect and would be superior to other non-sulfhydryl containing ACE inhibitors.[11] Additionally, the potential advantages of SH-group ACE inhibitors over the non-SH group in the improvement of endothelial dysfunction have been confirmed by the other study.[67] On the contrary, a previous study found that SH-containing compounds such as captopril, whether or not they possess ACE inhibitory activity, do not effectively scavenge superoxide radicals.[68]

Furthermore, in this study, TQ decreased TAC non-significantly and its combination with ZOF potentiated the improvement of the total capacity of the antioxidant system significantly. This finding is consistent with the effect of TQ in an experimental study of CPH-induced hemorrhagic cystitis, as TQ alleviated the oxidative stress induced by CPH via an increase in the level of antioxidant molecules reduced glutathione, and increased the activity of superoxide dismutase and catalase,[24] also TQ was able to increase antioxidant enzymes in carfilzomib-induced renal impairment.[59] Furthermore, it showed a potential synergistic effect with vitamin E in alleviating oxidative stress and improving the endogenous antioxidant system.[69]

Urinalysis parameters on dipstick tests 48 hours after CPH injections were assessed for screening hematuria, protein urea, and pus cells, the dipstick test has been used due to its superiority in terms of cost and sensitivity in the detection of hematuria.[70] Significant prevention of urothelium injury has been noticed through semiquantitative measurement of hematuria on the dipstick, measurement of pus cells, and protein urea in ZOF, TQ, and their combination-treated groups compared with the CPH-treated group. Hematuria in this finding was represented by images as well as by evaluating the scoring methods as stated in the previous studies.[29]

Moreover, in the current study, CPH disturbed the urinary excretion of urea, creatinine, and protein, there was a slight and non-significant increase in the urine concentration of urea, creatinine, and protein. In an experimental study, rats treated with CPH demonstrated lowered urine pH, a reduction in the urinary excretion of electrolytes, urea, and uric acid, and increased urine output,[71] also another study of CPH-induced cystitis, showed increased urine protein and urine output alongside decreased urinary urea and creatinine,[72] therefore inconsistent response on these parameters has been recorded in the literature. In this study, ZOF preserved the kidney function by reducing their excretion non-significantly. Various mechanisms elucidated the renoprotective effect of ACE inhibitors, such as captopril reduced urinary protein excretion in puromycin aminonucleoside nephrotic rats by enhancing intrarenal prostaglandin synthesis[73] or by reducing glomerular capillary pressure.[74] TQ alone and in combination with ZOF has no significant effect on these variables after CPH injection. Furthermore, the histopathological findings in this study supported the macroscopic and biochemical results and aligned with the observations reported in the previous studies, in which the renoprotective and antioxidant effects of ZOF have been demonstrated in diabetic rat models via the improvement of histopathological alteration in the kidney.[75] In another study, TQ mitigated the isoprenaline-induced histopathological changes in tubular epithelial cells and renal damage including renal interstitial hemorrhage that occurred in renal tissue by reducing fibrosis and inflammation.[76] Several limitations are present in this study, first; the selection of the dose of ZOF and TQ was based on the literature, therefore, there was no reduction of ZOF and/or TQ dose in the combined form. Second, the assessment of more novel biomarkers is necessary to elucidate the molecular mechanism and signaling pathways of sulfhydrylated-ZOF in protecting urinary tract urothelium.

Conclusion

The findings of this study demonstrated that ZOF and TQ exert significant uroprotective and nephroprotective effects against CPH-induced nephrotoxicity, with their combined use showing synergistic protective effects. These agents reduced the kidney-specific biomarkers including KIM-1 and Cys-C, attenuated pro-inflammatory cytokines such as IL-6 and TNF-α along with novel CBC-derived inflammatory biomarkers like NLR and NMR and restored TAC. Furthermore, these agents improved histopathological outcomes by reducing tissue degeneration and mitigating lesion scoring which supported the macroscopic and biochemical results. The suggested mechanism involves the anti-inflammatory and antioxidant activity of the sulfhydrylated ACE inhibitor- ZOF and herbal agent TQ. These findings emphasize the importance of further investigation to validate the mechanistic insight and the efficacy of these agents in clinical settings.

Data Sharing Statement

Data will be available upon request. The original data generated in the study are included in the article; further inquiries can be addressed to the corresponding author.

Ethical Approval

All the procedures in this study followed the standard principle of laboratory animal care and national institutional animal care. Additionally, the protocol of the study was approved by the Ethical and Research Registration Committee of the College of Pharmacy-University of Sulaimani with a registration number (PH99-23 on May 24th, 2023).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflict of interest.

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