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Research Article

Effects of Ertugliflozin and Lycopene on Ifosfamide-Induced Nephrotoxicity in Rats

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Abstract

Background: Nephrotoxicity induced by chemotherapy is a common side effect of many anticancer drugs. ***Objective***: To evaluate the effect of ertugliflozin (ERTU) and lycopene (LYCO) against ifosfamide (IFO)-induced nephrotoxicity. ***Methods***: 56 rats were divided into eight groups: negative control (NC), positive control (PC), control vehicle (CV), ERTU: ertugliflozin 20mg/kg, LYCO: lycopene 30mg/kg, IFO+ERTU, IFO+LYCO and IFO+ERTU+LYCO. On the 12th, 13th, and 14th days of the experiment, IFO 50 mg/kg was injected into PC, IFO+ERTU, IFO+LYCO, and IFO+ERTU+LYCO rats. Urine was collected for urinalysis. Blood and kidney tissue were harvested for oxidative stress, CBC-inflammatory and kidney injury biomarkers, and histopathological assessment. ***Results***: IFO resulted in hematuria and proteinuria, elevation of cystatin C, kidney injury molecule-1 (KIM-1) in kidney tissue, and a reduction in total antioxidant capacity (TAC). Platelet-to-lymphocyte ratio (PLR), platelet-to-monocyte ratio (PMR), and hemoglobin-to-lymphocyte ratio (HLR) increased significantly with histopathological alteration in kidney tissue. IFO+ERTU and IFO+ERTU+LYCO groups showed alleviation in the hematuria, proteinuria, and cystatin C. KIM-1 was significantly reduced in IFO+ERTU and non-significantly in IFO+LYCO and its combination. IFO+ERTU, IFO+LYCO, and IFO+ERTU+LYCO groups showed elevation in TAC. In IFO-exposed animals, ERTU resulted in a significant reduction in PLR and HLR and PMR non-significantly, and LYCO+ERTU significantly reduced PLR. ***Conclusions***: ERTU and LYCO alone and in combination alleviated kidney injury parameters and the histopathological lesions. These findings suggest that ERTU and LYCO are effective nephroprotective agents against IFO-induced nephrotoxicity. The suggested mechanisms are attributed to their antioxidant and anti-inflammatory actions for both diuretic and natriuretic properties for ERTU.

Keywords: Antioxidant, Cystatin C, Ertugliflozin, KIM-1, Lycopene.

تأثير إرتوغليفلوزين والليكوبين على السمية الكلوية التي يسببها إيفوسفاميد في الجرذان

الخلاصة

الخلفية: السمية الكلوية الناجمة عن العلاج الكيميائي هي أحد الآثار الجانبية الشائعة للعديد من الأدوية المضادة للسرطان. **الهدف**: تقييم تأثير إرتوغليفلوزين (ERTU) والليكوبين (LYCO) ضد السمية الكلوية الناجمة عن إيفوسفاميد (IFO). **الطرائق**: تم تقسيم 56 جرذا إلى ثماني مجموعات: التحكم السلبي (NC)، التحكم الإيجابي (PC)، المجموعة الضابطة (CV)، أرتوجليتازون (ERTU) 20 مجم/كجم، الليكوبين (LYCO) 30 مجم/كجم، IFO+LYCO، IFO+ERTU و IFO+ERTU+LYCO. في الأيام 12 و 13 و 14 من التجربة، تم حقن 50 IFO مجم/ كجم في جرذان PC و IFO+ERTU و IFO+LYCO و IFO+ERTU+LYCO. تم جمع البول والدم وأنسجة والكلى من أجل تحليل الإجهاد التأكسدي، والمؤشرات الحيوية للالتهاب CBC وتضرر الكلى، والتقييم النسيجي المرضي. **النتائج**: أدى IFO إلى بيلة دموية وبيلة بروتينية، وارتفاع السيستاتين C ، وجزيء إصابة الكلى-1 (KIM-1) في أنسجة الكلى، وانخفاض في القدرة الإجمالية المضادة للأكسدة (TAC). زادت نسبة الصفائح الدموية إلى الخلايا الليمفاوية (PLR) ، ونسبة الصفائح الدموية إلى الخلايا الوحيدة (PMR)، ونسبة الهيموجلوبين إلى الخلايا الليمفاوية (HLR) بشكل ملحوظ مع التغيير النسيجي المرضي في أنسجة الكلى. أظهرت مجموعات IFO+ERTU و IFO+ERTU+LYCO تخفيفا في البيلة الدموية والبيلة البروتينية والسيستاتين C. انخفض KIM-1 بشكل ملحوظ في IFO+ERTU وغير ملحوظ في IFO+LYCO ومزيجها. أظهرت مجموعات IFO+ERTU و IFO+LYCO و IF+ERTU+LYCO ارتفاعا في TAC. في المعرضة ل IFO ، أدى ERTU إلى انخفاض كبير في PLR و HLR و PMR بشكل غير كبير، وقلل LYCO + ERTU بشكل كبير من PLR. **الاستنتاجات**: خفف ERTU و LYCO لوحدهما أومجتمعين من معايير إصابة الكلى والآفات النسيجية. تشير هذه النتائج إلى أن ERTU و LYCO هما عوامل فعالة واقية ضد السمية الكلوية التي يسببها IFO. تعزى الآليات المقترحة إلى أفعالها المضادة للأكسدة والمضادة للالتهابات لكل من الخصائص المدرة للبول والصوديوم ل ERTU.

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INTRODUCTION

Nephrotoxicity induced by chemotherapy is a common side effect of many anticancer drugs such as cyclophosphamide (CYP) and ifosfamide (IFO) [1,2]. The hepatic and extrahepatic biotransformation of both CYP and IFO leads to the generation of by-products, such as acrolein and chloroacetaldehyde, which contribute to the toxicities of these drugs [3]. Acrolein is a highly reactive unsaturated aldehyde that disrupts cellular redox balance, leading to the generation of reactive oxygen species (ROS) and

depletion of glutathione [4]. Chloroacetaldehyde, like acrolein, contributes to severe oxidative stress and depletion of reduced glutathione, which reduces the cells' capacity to scavenge free radicals, therefore increasing their susceptibility to oxidative stress and decreasing cellular adenosine triphosphate (ATP) levels [5]. This byproduct induces nephrotoxicity by disrupting oxidative phosphorylation and inhibiting gluconeogenesis in kidney cortical cells; the resulting oxidative stress plays a role in renal dysfunction [6]. Ifosfamide can cause both tubular and glomerular damage, with the risk increasing at higher cumulative doses [7]. Proximal tubular injury is the most common and severe complication. Furthermore, the role of acrolein in catalyzing the production of reactive oxygen and nitrogen species and superoxide radicals in the urothelium cannot be excluded; this is leading to membrane and DNA damage and ultimately causing cell death through the nuclear factor kappa-B (NF-κB) pathway [8]. Numerous in-vitro, in-vivo, and clinical efforts have been made to minimize the nephrotoxicity associated with IFO; however, none have been entirely effective or without adverse effects. As a result, repurposing already approved medications that possess established safety profiles and cost-effectiveness have emerged as a promising strategy to address these challenges. For example, simvastatin has demonstrated promise in preventing IFO-induced nephrotoxicity by decreasing plasma creatinine levels and lipid peroxidation in animal studies [9]. Sodium-glucose co-transporter 2 (SGLT2) inhibitors represent one of the new classes of medications for managing type 2 diabetes mellitus (T2DM) by preventing glucose reabsorption in the kidneys. Besides their ability to lower blood glucose levels, these antidiabetic medications may also have beneficial effects on the cardiovascular and renal systems [10]. In recent years, SGLT2 inhibitors have demonstrated potential in mitigating cisplatin-induced kidney damage. These drugs have been found to enhance renal function, lower oxidative stress, and reduce inflammation. Moreover, SGLT2 inhibitors have been shown to decrease the accumulation of the chemotherapeutic agent in the kidneys, which is believed to be a key mechanism behind the attenuation of chemotherapy-induced nephrotoxicity [11]. The SGLT-2 inhibitors have anti-inflammatory and antioxidant actions [11,12]. Since inflammation and oxidative stress are key mechanisms in IFO-induced nephrotoxicity, a promising strategy to mitigate its toxicity, particularly nephrotoxicity, may involve the use of antioxidants and anti-inflammatory agents. We hypothesized that ERTU, which has not been investigated yet, will be an effective candidate for amelioration of IFO-induced nephrotoxicity via these mechanisms. On the other hand, researchers have highlighted the potential effect of various natural herbal products in reducing chemotherapy-induced nephrotoxicity [13]. A recent study demonstrated that pretreatment with ellagic acid offers protection against IFO-induced nephrotoxicity by maintaining mitochondrial function and mitigating oxidative stress [14]. These herbal compounds primarily function by alleviating oxidative stress, suppressing inflammatory cytokines, and maintaining bladder tissue integrity. Lycopene (LYCO), a carotenoid found in tomatoes, has been shown to have nephroprotective effects in multiple studies. Evidence indicates that LYCO can alleviate oxidative stress and inflammation in the kidneys induced by various agents [15]. In an experimental mouse model, LYCO has a nephroprotective effect against renal ischemic reperfusion injury [16]. The antioxidant properties of LYCO are critical in counteracting the oxidative stress induced by CYP, as shown by reduced malondialdehyde levels and increased activity of antioxidant enzymes. LYCO, when combined with melatonin, has its protective effects against CYP-induced toxicity further enhanced [17]. These findings suggest that LYCO possesses therapeutic potential in preventing IFO-induced uro- and nephrotoxicity. Today, there is growing support for adjuvant use of herbal medicine with nephroprotective agents for the improvement of urinary and renal function. The antioxidant and anti-inflammatory characteristics of both the SGLT2 class and LYCO seem integral to their pleiotropic therapeutic benefits in treating various conditions linked to oxidative stress and inflammation, including chemotherapy-induced nephrotoxicity. Therefore, this study aimed to explore the potential nephroprotective effects of ERTU and LYCO in IFO-induced nephrotoxicity in albino rats through the assessment of specific kidney function biomarkers, oxidative stress parameters, and histopathological alterations in the kidney tissue.

METHODS

Chemicals and drugs

Ifosfamide was obtained from Hikma Pharmaceuticals, Jordan, and ertugliflozin was from SAMI Pharmaceuticals, Pakistan. Lycopene was from Pure Encapsulations, USA. Xylazine and ketamine were from BELTAVETFARMA, Turkey, and DOĞA ILAÇ, Turkey, respectively. Rat Total Antioxidant Capacity (TAC), Cystatin C, and Kidney Injury Molecule 1 (KIM-1) ELISA kits were from Bioassay Technology Laboratory BT LAB, Shanghai, China. The urine reagent strip for urinalysis was from CYBOW, Republic of Korea. Complete blood count was measured using Hematology Analyzer Mindray BC-2800 Vet, Veterinarian Hematology Analyzer, China.

Ethical statement

The protocol of this study was approved by the ethical committee of the College of Veterinary Medicine-University of Sulaimani with registration number VET 0238 on 27.11.2024. All procedures were performed following the Principle of Laboratory Animal Care.

Experimental design and treatment protocol

A total of 56 Wistar Albino male rats of 10-12 weeks (weighing 180 ± 20 g) were used for the study; they

were obtained from the animal house of the University of Tikrit. The rats were acclimatized in a 12-hour light-dark cycle, a temperature of 22 ± 1 °C, and a relative humidity of 50 ± 5% for 2 weeks. Figure 1 shows the study design.

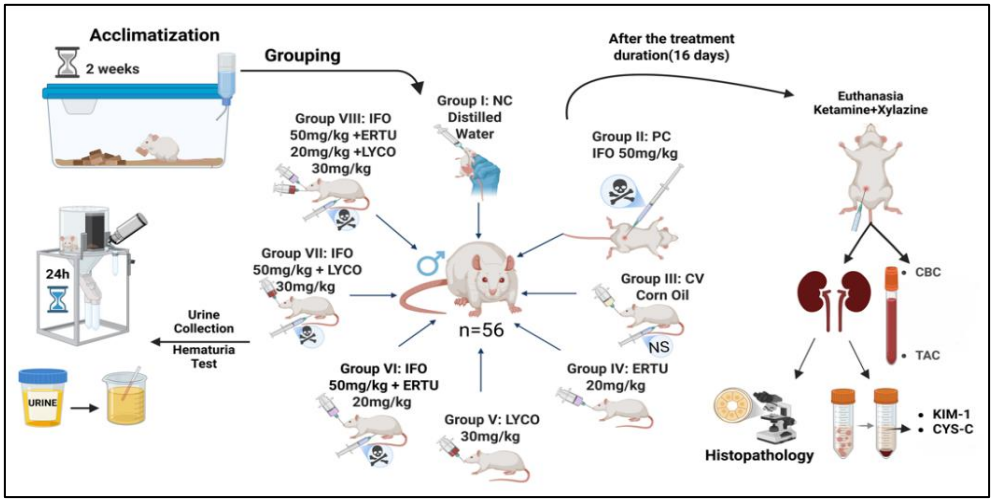

Figure 1: Study design, animal groups, and treatment protocol. TAC; total antioxidant capacity, KIM-1; kidney injury molecules-1, CYS-C; cystatin C, IFO; ifosfamide, NC; negative control, PC; positive control, CV; control vehicle, ERTU; ertugliflozin, LYCO; lycopene, CBC: complete blood count, n; number or rats.

The animals were divided into eight groups, each of seven rats, and the treatments were given for 16 days, as follows: The negative control (NC) received 1.0 ml of distilled water; the positive control (PC) received saline with IFO 50 mg/kg IP; the control vehicle (CV) group received corn oil; the ertugliflozin (ERTU) group received ERTU 20 mg/kg orally; the lycopene (LYCO) group received LYCO 30 mg/kg orally; the IFO + Ertugliflozin (IFO + ERTU) group received ERTU 20 mg/kg/day orally with IFO 50 mg/kg intraperitoneally (IP); the IFO + Lycopene (IFO + LYCO) group received LYCO 30 mg/kg/day orally with IFO 50 mg/kg IP; and the IFO + Ertugliflozin with Lycopene (IFO + ERTU + LYCO) group received ERTU 20 mg/kg with LYCO 30 mg/kg orally with IFO 50 mg/kg IP. All the treatments were given for 16 days and the dose of IFO 50 mg/kg IP was given in three consecutive doses on the 12^{th}, 13^{th}, and 14^{th} days of the experiment according to the above protocol. The dose and duration of IFO [9,18] ERTU, [19] and LYCO [17,20] were chosen based on the previous studies with modification.

Measurement of body weight and collection of urine and blood

The body weight of the rats was measured every week. The relative organ weight was calculated using the following formula: Relative organ weight = [organ weight/body weight] × 100. Following the last dose of IFO injection, the rats were put in metabolic cages, and urine was collected over 24 hours for urine analysis using a dipstick test. After 48 hours of the last dose of IFO injection, the animals were sacrificed, and approximately 5 ml of blood was collected from the caudal vena cava. A portion of the collected blood was placed in an ethylenediaminetetraacetic acid (EDTA) tube for complete blood count (CBC) analysis, while the remainder was transferred to a serum separator tube and centrifuged at 3000 rpm for 20 minutes at 4°C. The resulting serum was then stored at −80°C for subsequent biochemical analysis.

Tissue collection and homogenization

Both kidneys were excised through careful dissection. One kidney was homogenized, and the resulting homogenate served for the assessment of kidney-specific biomarkers. The second kidney was rinsed with ice-cold normal saline and fixed in 25 mL of formaldehyde 10% for histopathological evaluation.

Measurement of biomarkers

Serum was used for the measurement of TAC, and the CBC-derived inflammatory biomarkers, including PLR, PMR, and HLR, were calculated from CBC parameters. Kidney tissue homogenate was used for measurement of the kidney-specific biomarkers, cystatin C and KIM-1.

Macroscopic and histological analysis

Histological changes in the kidney were investigated using histopathologic scoring. Hematuria was evaluated in the urine samples collected in all groups 24 hours after the IFO injection. The dipstick test [21] 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, and 4 = macroscopically detectable hematuria. This test has been used based on the previous studies [13,22]. Following euthanasia, necropsy was performed to collect tissue samples for histological analysis. The kidney samples were stabilized and placed into tissue cassettes, then fixed in 10% neutral buffered formalin for approximately 48 hours. Next, the tissue sections were dehydrated by passing through a graded ethanol series (50%, 60%, 70%, 90%, and 100%), followed by three xylene

clearing steps. Subsequently, the processed samples were infiltrated and embedded in molten paraffin blocks at a temperature of 60–70°C using an automated wax embedder. The paraffin-embedded tissues were then sectioned into 5 µm slices using a semi-automated rotary microtome. The prepared tissue sections were mounted on glass slides and dried on a hot plate. To remove paraffin, the slides were treated with xylene for 30 minutes, then dried in a hot oven at 50°C for 5 minutes. Finally, the slides were cleaned with xylene, cover-slipped, stained with Harris's hematoxylin and eosin solution, and examined under a bright-field light microscope.

Histopathologic scoring

The following parameters or lesions were assessed to score and grade the pathologic alteration in the kidney: Grade 0 refers to renal tubules and glomeruli that are not altered. Grades 1-4 consist of congestion with interstitial hemorrhage, renal glomeruli, and tubular epithelial cell degeneration without appreciable necrosis [23] with modification. Grade 1: Mild change involving up to 25%; initiation of change. Grade 2: Moderate change involving 26–50%. Grade 3: Moderate-severe change involving 51–75%. Grade 4: Severe change involving ≥75%; widespread changes identified.

Ethical consideration

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 Veterinary Medicine at the University of Sulaimani with registration number VET 0238 on 27.11.2024.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism software version 10.4.1. The Shapiro-Wilk test was used to test the distribution of the variables. For normally distributed data, parametric analysis was performed using a one-way ANOVA test followed by Tukey's multiple comparisons for comparison between different groups. While the Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for non-parametric values. Two-way ANOVA multiple comparisons followed by Bonferroni or Sidak tests were used for repeated measures such as body weight. Descriptive statistics was used to calculate mean ± SEM. A p-value < 0.05 was considered statistically significant.

RESULTS

All groups showed normal weight gain with no significant differences between different treated groups (p> 0.05) at each time interval, as shown in Figure 2A. Relative kidney weight was comparable in all treated groups with the PC group except in the animals in the IFO+ERTU+LYCO group, which showed a significant increase in relative organ weight (p< 0.025). Comparable results were observed in CV, ERTU, and LYCO with the NC group (p> 0.05), as shown in Figure 2B.

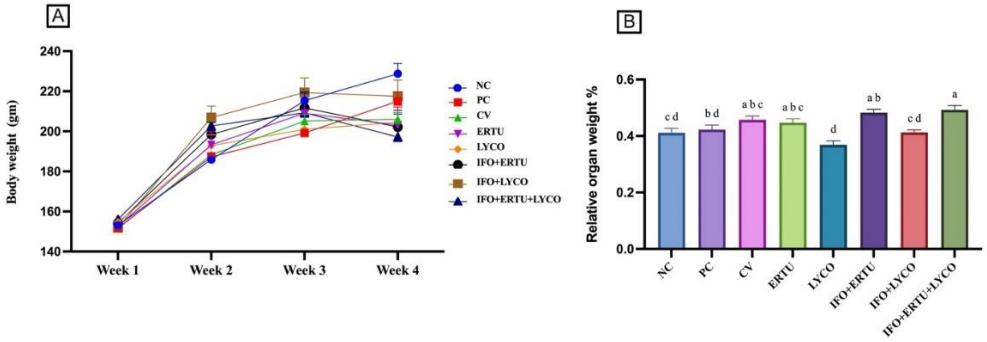

Figure 2: Effect of ertugliflozin and lycopene alone or in combination on **A)** body weight, **B)** Relative organ weight in IFO-induced nephrotoxicity. Data are presented as mean ± SEM, n=7. Data in A was analyzed by Two-way ANOVA multiple comparison followed by Bonferroni test, while in B by one-way ANOVA followed by Tukey's test. p<0.05 is considered statistically significant. NC: negative control, PC: positive control, CV: control vehicle, ERTU: ertugliflozin, LYCO: lycopene, IFO: ifosfamide. Non-identical letters (a,b,c,d) indicate statistically significant differences between different groups.

However, a significant increase in the relative organ weight was observed in the IFO+ERTU and IFO+ERTU+LYCO groups *vs*. the NC (p= 0.02, p= 0.004, respectively). A macroscopic examination of the urine for hematuria, proteinuria, and urine volume was conducted using a dipstick test (Figure 3 A-F). The representative images of dipstick tests for hematuria, proteinuria, and gross hematuria for the color of the urine in PC and the other groups are shown in Figure 3 A-C. Hematuria and proteinuria were markedly noticed in the PC group in comparison to the NC group (p= 0.0005, p= 0.0007, respectively), as shown in Figure 3 D and E. Pretreatment of the IFO-exposed rats with ERTU and ERTU+LYCO significantly reduced RBCs in the urine and alleviated the hematuria (p=0.0063, p=0.0039, respectively). Meanwhile, LYCO in IFO+LYCO non-significantly decreased the hematuria (p> 0.05). Additionally, pretreatment of IFO-exposed rats with ERTU, LYCO, and their combination in IFO+ERTU, IFO+LYCO, and IFO+ERTU+LYCO groups non-significantly decreased proteinuria compared to the PC group (p>

0.99). IFO injections resulted in a significant reduction in the urine volume in the PC group when compared to the rats in the NC group (p= 0.003), as shown in Figure 3F.

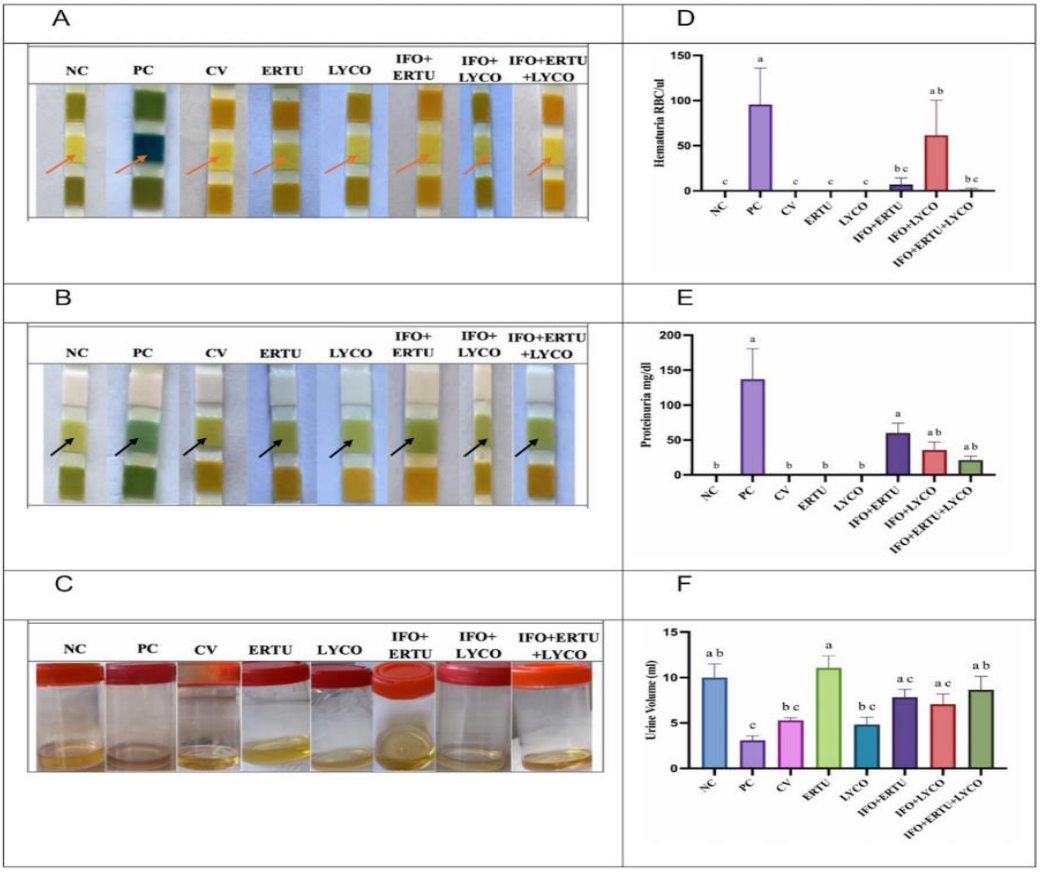

Figure 3: Representative images of dipstick tests for **A**) Hematuria; red arrows show the area of hematuria detection in the dipstick test; **B)** Proteinuria; black arrows show the area of proteinuria in the dipstick test; **C)** Gross hematuria; urine color visibly red or brown in the PC group; **D-F)** Bars represent scores of **(D)** hematuria **(E)** proteinuria, and **(F)** urine volume**.** Data presented as mean ± SEM, n=7, data analyzed by Kruskal–Wallis test for non-parametric data followed by Dunn'post-hoc test. While one-way ANOVA multiple comparisons followed by Tukey's test for urine volume. p<0.05 is considered statistically significant. NC: negative control, PC: positive control, CV: control vehicle, ERTU: ertugliflozin, LYCO: lycopene, IFO: ifosfamide. Non-identical letters (a,b,c,d) indicate statistically significant differences between different groups.

Comparable urine output was observed in rats treated with ERTU alone (p> 0.05) compared to NC; however, a non-significant reduction in urine volume was obtained in CV and LYCO groups (p> 0.05) versus NC. Pre-treatment of IFO-exposed rats with ERTU and LYCO in IFO+ERTU and IFO+LYCO groups resulted in improvement of urine output in a non-significant manner (p= 0.097, p= 0.2, respectively) in comparison to PC; meanwhile, their combination increased urine output significantly (p= 0.021). In the current study, water consumption (ml/rat/24 hours) by the animals was monitored. The average volume of water consumption (ml/rat/24 hours) was increased significantly in all ERTU-treated groups before and after IFO injection (p< 0.0001) in comparison to PC (15.47 ± 1.12) and other treated groups as follows: ERTU= 26.83 ± 1.05, IFO+ERTU= 24.09 ± 0.86, and IFO+ERTU+LYCO= 24.92 ± 1.05, and water consumption in the NC (16.35 ± 1.17), CV (14.25 ± 0.55), and LYCO (13.26 ± 0.94) were comparable. The effect of ERTU and LYCO alone or their combination on the glomerular function biomarkers (cystatin C) and kidney injury (KIM-1) in IFO-induced nephrotoxicity is demonstrated in Figure 4 (A and B). IFO resulted in a significant elevation of the level of cystatin C in kidney tissue homogenate in comparison to NC groups (p= 0.0004) (Figure 4A). However, pre-treatment with ERTU in the IFO+ERTU group protected the kidney from the injury associated with IFO injection significantly (p< 0.0001). Pre-treatment of LYCO in the IFO+LYCO group resulted in a non-significant decrease in cystatin C level (p= 0.19) compared to PC. Meanwhile, its combination with ERTU in IFO+ERTU+LYCO significantly reduced the level of this specific kidney function biomarker (p< 0.0001) in comparison with PC. The level of cystatin C in the vehicle, ERTU, and LYCO groups was parallel to the NC group (p> 0.05) exhibiting no harmful effect in the experiment. In Figure 4B, IFO resulted in a significant elevation of KIM-1 in the tissue homogenate compared to NC (p= 0.0039). The level of KIM-1 in CV, ERTU, and LYCO groups was comparable to NC (p> 0.05). This indicates an inert and safe nature of the vehicle and the tested agents in the experiment. Administration of ERTU protected the kidney from IFO-induced nephrotoxicity significantly (p= 0.04). Pre-treatment of LYCO and its combination with ERTU restored the KIM-1 level in a non-significant manner (p> 0.05). The antioxidant status of all groups was examined via measurement of TAC. IFO injections resulted in a reduction in the serum level of TAC (p> 0.05) non-

significantly in comparison to NC and other control groups (Figure 4C). Pre-treatment of ERTU and its combination with LYCO elevated the TAC level non-significantly, while LYCO in the IFO+LYCO group elevated TAC significantly (p= 0.0084).

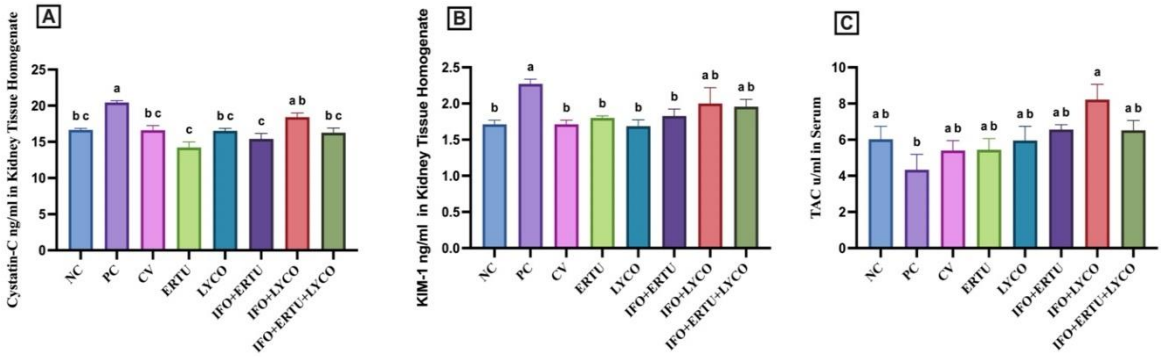

Figure 4: Effect of ertugliflozin and lycopene alone or in combination on **A)** Cystatin C, **B)** KIM-1, and **C)** TAC in IFO-induced nephrotoxicity. Data are presented as mean ± SEM, n=7, data analyzed by one-way ANOVA multiple comparisons followed by Tukey's test. p<0.05 is considered statistically significant. NC: negative control, PC: positive control, CV: control vehicle, ERTU: ertugliflozin, LYCO: lycopene, IFO: ifosfamide, KIM-1: kidney injury molecule -1, TAC: total antioxidant capacity. Non-identical letters (a,b,c,d) indicate statistically significant differences between different groups and versus PC.

The current study applied CBC-derived inflammatory biomarkers to follow up on the inflammatory process of the animals that received different treatments, as shown in Figure 5 (A-C). The values of PLR, PMR, and HLR showed a significant increase in PC animals in comparison to the NC group (p= 0.0002, p= 0.0029, and p< 0.0001, respectively), as shown in Figure 5 (A-C). The values of PLR, PMR, and HLR in rats in CV, ERTU, and LYCO were comparable with NC groups with a non-significant difference (p< 0.05) indicating no significant role of these substances in the alteration of these CBC-derived inflammatory biomarkers. Pre-treatment of the animals with ERTU and its combination with LYCO in IFO-induced nephrotoxicity groups resulted in a significant reduction in the value of PLR in comparison to the PC group (p= 0.01, p= 0.017, respectively); meanwhile, LYCO pre-treatment has a non-significant effect in mitigation of PLR (p> 0.9) as shown in Figure 5A. Pre-treatment of the animals with ERTU, LYCO, and their combination in IFO+ERTU, IFO+LYCO, and IFO+ERTU+LYCO groups resulted in a decrease of PMR non-significantly (p> 0.05) as shown in Figure 5B. Meanwhile, ERTU significantly decreased HLR compared to IFO-exposed animals in the PC group (p= 0.034). While animals in IFO+ERTU+LYCO showed a non-significant reduction in the HLR value (p= 0.1). LYCO was entirely unable to restore HLR in the IFO+LYCO group (Figure 5C).

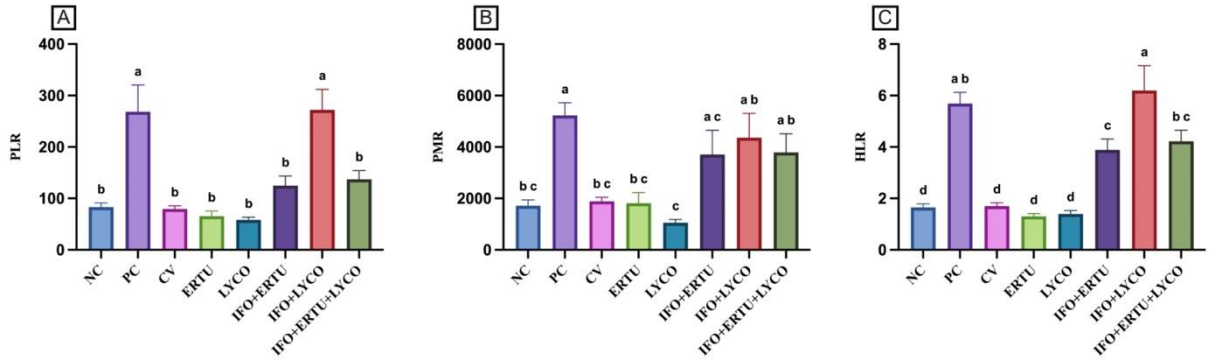

Figure 5: Effect of ertugliflozin and lycopene alone or in combination on CBC-derived inflammatory biomarkers in IFO-induced nephrotoxicity. **A)** PLR, **B)** PMR and **C)** HLR. Data are presented as mean ± SEM, n=7, data analyzed by one-way ANOVA multiple comparisons followed by Tukey's test. p<0.05 is considered statistically significant. NC: negative control, PC: positive control, CV: control vehicle, ERTU: ertugliflozin, LYCO: lycopene, IFO: ifosfamide, PLR: Platelet-to-lymphocyte ratio, PMR; platelet-to-monocyte ratio, HLR; hemoglobin-to-lymphocyte ratio. Non-identical letters (a,b,c,d) indicate statistically significant differences between different groups and versus PC.

Histopathologic analysis in the current study is shown in Figures 6, 7, and 8, and its histomorphometric analysis in Figure 9. The kidney section in the rat of the NC group revealed the normal architecture of the renal cortex and medulla and intact glomeruli structures (Figure 6 a-d). The PC group revealed marked lesions (Figure 6 e-l), severe degeneration of the renal corpuscle with marked degeneration and necrosis of the proximal and distal convoluted tubules and also marked infiltration of neutrophils seen in the interstitial and particularly surrounding the glomeruli (Figure 6 J and k). In comparison to the NC, the urothelium also revealed sloughing and degeneration of the lining mucosa with vascular congestion (Figure 6L). The kidney section that was treated with corn oil only (CV) also showed normal histologic structures of the nephron from the cortex to the urothelium, like the NC group (Figure 6 m-p).

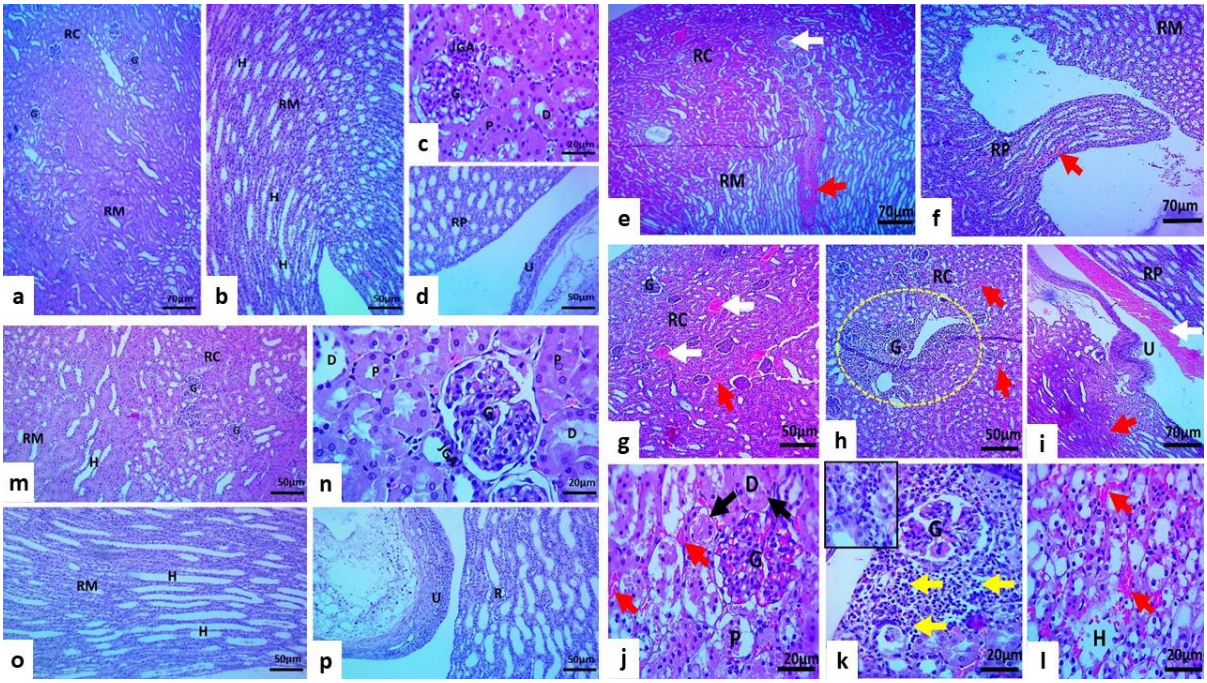

Figure 6: Microscopic sections of the kidney in control groups showed: **a-d**) Normal histologic organization of renal cortex and medulla in the negative control group. **e-i**) Marked vascular congestion (white arrows) and interstitial hemorrhage (red arrows) seen throughout renal parenchyma. c and d: h: Focal marked inflammatory reaction (yellow dash lines). i: Sloughing and degeneration of the urothelium. **j-l**) Severe degeneration of renal corpuscles with typical necrotic features of convoluted tubules (black arrows), severe neutrophil infiltration (inset and yellow arrows) with hemorrhage, and marked degeneration of collecting tubules and Henle loops with interstitial hemorrhage as indicated by red arrows in positive control. **m-p**) Normal histologic structures of renal cortex and renal medulla in a vehicle control group, (H&E stain); RC: Renal cortex, RM: Renal medulla, G: Glomeruli, P: Proximal convoluted tubules, D: Distal convoluted tubules, H: Henle loops, RP: Renal papillae, U: Urothelium.

In microscopical sections, the group treated only with ERTU revealed lesions only in the cortex; mild swelling in the renal corpuscle *vs.* the proximal and distal convoluted tubules that had mild-moderate swelling of lining epithelium, loss of brush border, and narrowing of the lumen, while the renal medulla, papillae, and urothelium showed normal structures (Figure 7 a-d). The sections of the kidney in the LYCO-treated group showed normal histologic structures of organized renal layers, including the renal cortex, medulla, papillae, and urothelium (Figure 7 e-h), while the glomeruli and proximal and distal convoluted tubules lining epithelium revealed the mild degree of swollen features of pale cytoplasm with centrally located nuclei (Figure 7g).

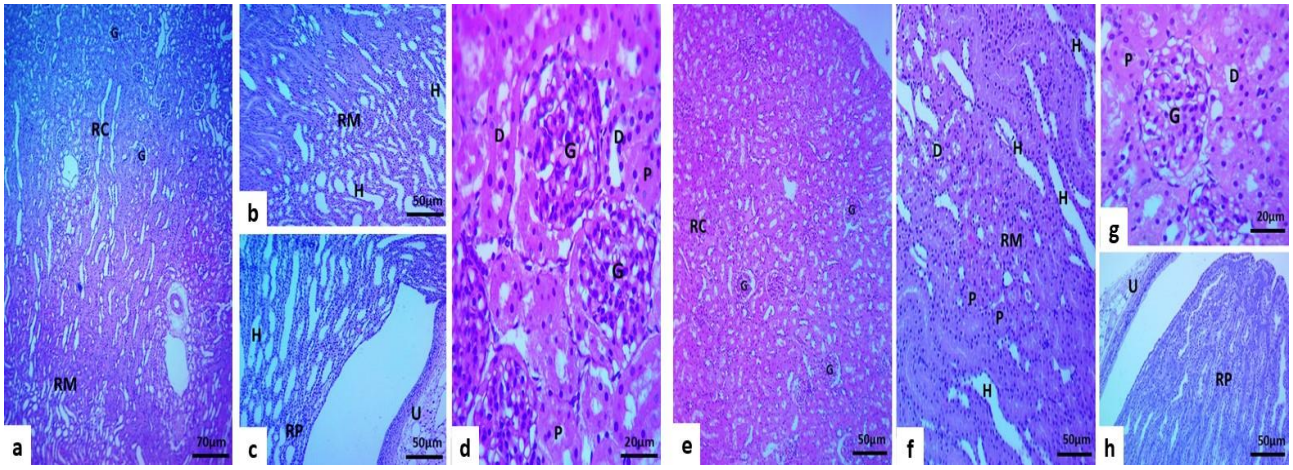

Figure 7: Microscopic sections of the kidney in the treated groups presented: **a-c**) Normal histological features of the renal cortex, medulla, and renal papillae with intact urothelium. d: Mild-moderate swollen of glomeruli with proximal and distal convoluted tubules lining epithelium in the Ertugliflozin-treated group. **e-h**) Normal histologic structures of renal cortex and medulla with intact renal papillae and urothelium, in section g, there were mild swollen glomeruli with proximal and distal convoluted tubules lining epithelium in Lycopene-treated group, (H&E stain). G: glomeruli, P: Proximal convoluted tubules, D: Distal convoluted tubules, H: Henle loops, RP: Renal papillae, U: Urothelium.

The administration of ERTU in the IFO+ERTU group alleviated the pathological alteration in the renal sections and reduced the severity to a moderate-mild degree (Figure 8 a-f). Additionally, the administration of LYCO in the IFO+LYCO group attenuated pathologic changes to mild interstitial hemorrhage in the renal cortex and medulla with mild degeneration of proximal and distal convoluted tubules and Henle loops (Figure 8 g-k), in comparison to the administration of a combination protocol of

ERTU+LYCO with IFO that attenuated the lesion to a mild degree and revealed mild interstitial hemorrhage in the renal cortex and medulla with mild degeneration of proximal and distal tubules, collecting tubules, and Henle loops. Also, renal papillae and urothelium showed normal features (Figure 8 l-q). The histomorphometric analysis of renal abnormalities among studied groups is presented in Figure 9A-I. The glomerular degeneration, proximal and distal tubular degeneration and necrosis, interstitial hemorrhage, vascular congestion, and inflammation peaked in the PC group.

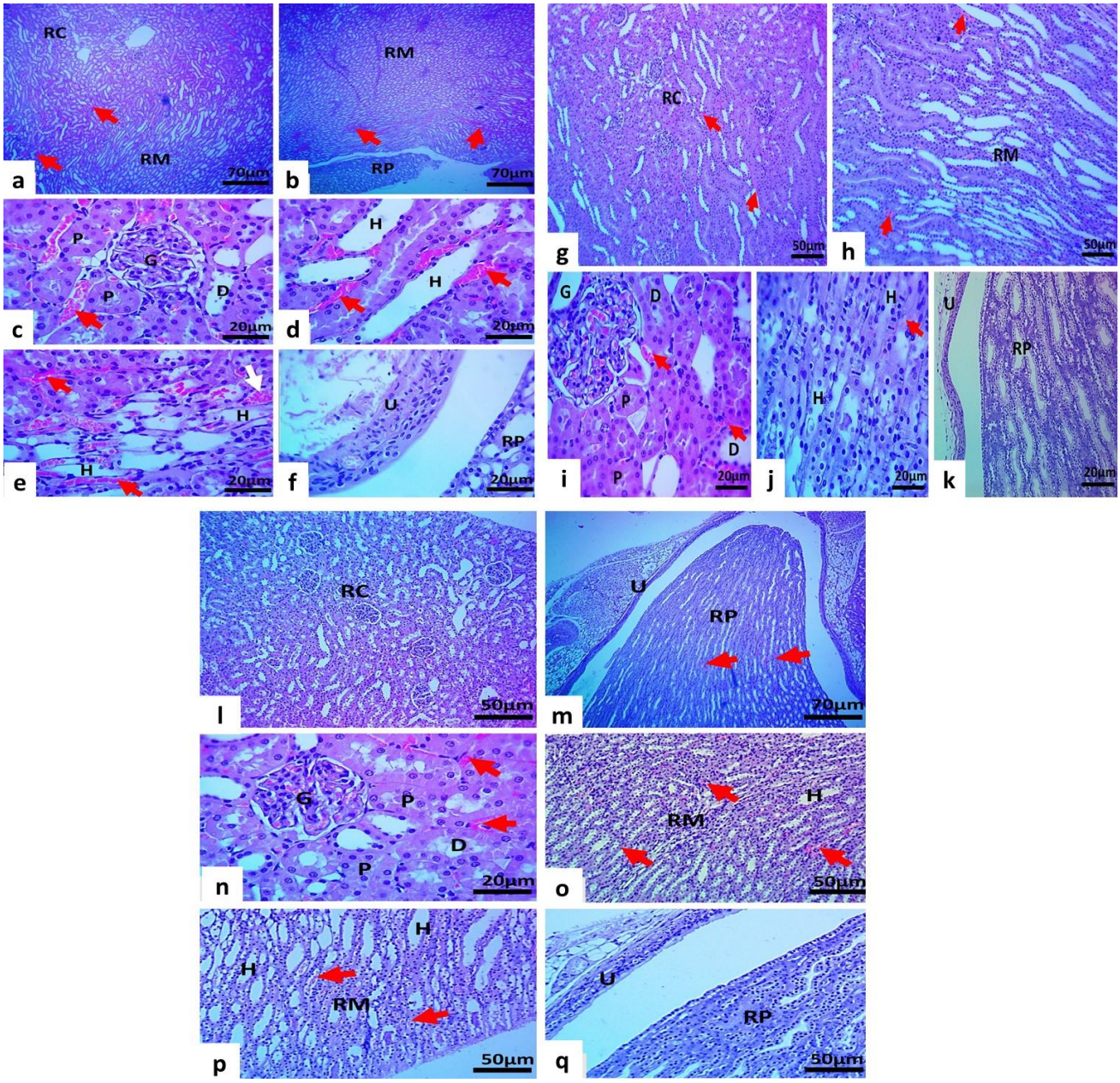

Figure 8: Microscopic sections of the kidney in IFO-treated groups displayed: **a-e**) Moderate vascular congestion (white arrows) and interstitial hemorrhage (red arrows) in the renal parenchyma, with mild degeneration of collecting tubules and Henle loops. **F**) Intact renal papillae and urothelium in the IFO+ERTU group. **g-j**) Mild interstitial hemorrhage (red arrows) in the renal cortex and medulla with mild degeneration of collecting tubules and Henle loops. k: Intact renal papillae and urothelium in the IFO+LYCO group. **l-p**) Mild interstitial hemorrhage (red arrows) in the renal cortex and medulla with mild degeneration of collecting tubules and Henle loops. **q**) Intact renal papillae and urothelium in IFO+ERTU+LYCO group, (H&E stain). RC: Renal cortex, RM: Renal medulla, G: Glomeruli, P: Proximal convoluted tubules, D: Distal convoluted tubules, H: Henle loops, RP: Renal papillae, U: Urothelium.

Mild or no alteration of the kidney histological structure was observed in the corn oil, ERTU, and LYCO-only treated groups. However, treatment with ERTU and LYCO, both individually and in combination, attenuated the lesions induced by IFO in a significant manner ($p< 0.05$) (Figure 9 A-F) and resulted in a reduction in overall lesion severity. Additionally, degeneration in collecting tubules, renal papillae, and urothelium induced by IFO was significantly ($p< 0.05$) alleviated in the treated groups of ERTU, LYCO, and their combination protocol ERTU+LYCO (Figure 9 G-I). The most pronounced protective effect was observed in the combination group (ERTU and LYCO), where lesion severity was significantly reduced from a severe to no or mild grade.

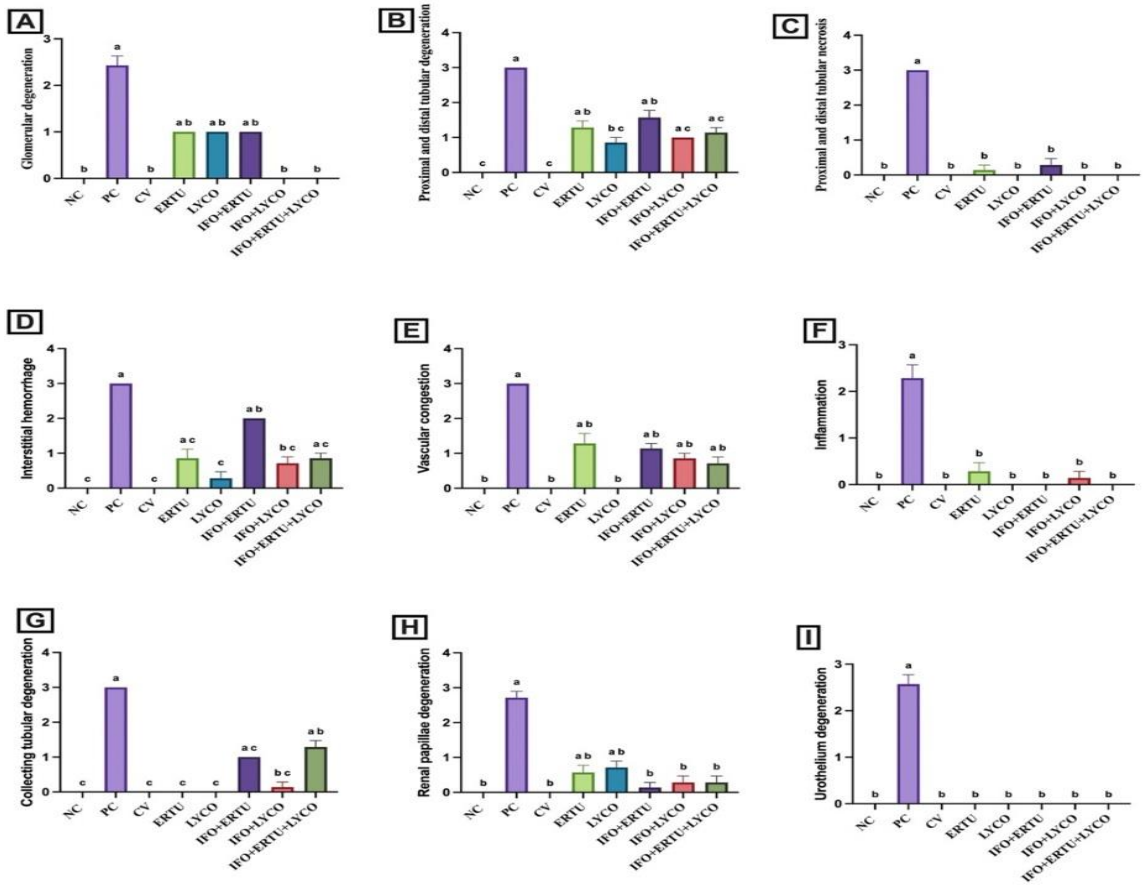

Figure 9: Histomorphometric analysis of renal abnormalities among studied groups. **A)** glomerular degeneration, **B)** proximal and distal tubular degeneration, **C)** proximal and distal tubular necrosis, **D)** interstitial hemorrhage, **E)** vascular congestion, **F)** inflammation, **G)** collecting tubular degeneration, **H)** renal papillae degeneration, **I)** urothelium degeneration. NC: negative control, PC: positive control, ERTU: ertugliflozin, LYCO: lycopene, IFO: ifosfamide. Kruskal Wallis multiple comparison followed by Dunn's test was used to determine the statistical differences between different groups. $p<0.05$ is considered as statistically significant.

Table 1: Interpretation of scoring and grading for different parameters of kidney lesions [24].

Locations	Histopathologic abnormalities	Scores	Grade	Interpretation
Glomerulus	Inflammation	0	No	Absence of Change
		1	Mild	Change in less than 25%
		2	Moderate	Change in 26-50%
		3	Moderate-Severe	Change in 51-75%
		4	Severe	Changes ≥ 75%
	Degeneration/Necrosis	0	No	Absence of Change
		1	Mild	Change in less than 25%
		2	Moderate	Change in 26-50%
		3	Moderate-Severe	Change in 51-75%
		4	Severe	Changes ≥ 75%
Tubular compartment	Cellular swelling or Hydropic degeneration	0	No	Absence of Change
		1	Mild	Change in less than 25%
		2	Moderate	Change in 26-50%
		3	Moderate-Severe	Change in 51-75%
		4	Severe	Changes ≥ 75%
	Necrosis	0	No	Absence of Change
		1	Mild	Change in less than 25%
		2	Moderate	Change in 26-50%
		3	Moderate-Severe	Change in 51-75%
		4	Severe	Changes ≥ 75%
Interstitial compartment	Congestion and hemorrhage	0	No	Absence of Change
		1	Mild	Change in less than 25%
		2	Moderate	Change in 26-50%
		3	Moderate-Severe	Change in 51-75%
		4	Severe	Changes ≥ 75%

DISCUSSION

The present study explored the repurposing of a clinically approved SGLT2 inhibitor, ERTU, alongside an herbal medicine, LYCO, in an experimental model of IFO-induced nephrotoxicity. The principal findings of this study demonstrated a substantial nephroprotection reflected by various renal-attributed parameters, including new protein biomarkers, physiological parameters, and

histological analysis. In the current study, IFO was used to induce nephrotoxicity in rats. IFO-nephrotoxicity was evidenced by macroscopic, microscopic, and biochemical parameters (Table 1) [24]. Gross hematuria and proteinuria were markedly noticed via dipstick test in the rat's urine of the IFO-exposed group. Consistent with these findings, both earlier and recent research have shown that administering IFO at a dose of 50-80 mg/kg for three to five consecutive days leads to marked kidney damage [18,25]. In the assessment of the living parameters, no significant changes were observed in the body weight of IFO-exposed animals compared to the other groups. Comparable results from a previous study showed that treating rats with IFO and LYCO had no significant effect on body weight [26]. SGLT2 inhibitors have been shown to reduce body weight by increasing urinary excretion of glucose and calorie loss [27]. An observational clinical study found that taking SGLT2 inhibitors reduced body weight due to increased urine output, fluid loss, a slight decrease in plasma volume, and a temporary decrease in extracellular fluid [28]. In the present study, ERTU exhibited a non-significant effect on the weight of the animals. The reason for this finding could be attributed to higher water consumption as a compensatory mechanism to counteract osmotic diuresis. Relative kidney weight also was comparable in all treated groups when compared with the PC group, except in the IFO-exposed animals treated with ERTU+LYCO, which showed a significant increase in relative organ weight. This might be related to the impact of ERTU, which may cause osmotic swelling in the cells of the renal tubule and corpuscle, resulting in increased kidney weight. This finding agrees with an earlier study, which revealed that fluid-induced cell swelling is responsible for empagliflozin-induced kidney weight gain [29]. Urine volume measurements among the study groups provide valuable insights into kidney function under the effect of IFO, ERTU, and LYCO. In the current study, IFO significantly decreased urine output as compared to the negative control. This outcome contradicts a clinical study, which found that IFO triggered polyuria through antidiuretic hormone resistance [30]. This reduction in urine output in the current study could be attributed to the IFO-induced glomerular injury, which causes a drop in glomerular filtration rate (GFR) and urine production. However, both ERTU and LYCO increased urine production, but the combined protocol was more effective in restoring urine volume. ERTU blocks SGLT2 in the proximal renal tubule, which lowers glucose and sodium reabsorption. This results in glucosuria, osmotic diuresis, and an increase in urine output. The finding agrees with previous research conducted by Patel *et al.* [31] which demonstrated profound urination in SGLT2 inhibitor users. Additionally, the current findings are consistent with an earlier investigation, which indicated that the administration of LYCO enhances urine output in ethylene glycol-induced nephrolithiasis [32]. The antioxidant activity of LYCO may support the rise in urine volume. In the present study, significant glomerular and tubular degeneration was observed in the histopathologic findings of the IFO-exposed group. This finding is comparable with the results obtained in the previous studies, which demonstrated a profound histopathological alteration after IFO injection [14]. In this study, the animals pretreated with ERTU and LYCO alone and in their combination alleviated the lesions induced by IFO significantly. A previous study conducted by Abdelrahman et al. has consistently shown that canagliflozin alleviated histopathological alteration induced by the chemotherapeutic agent cisplatin through attenuation of inflammation and oxidative stress [11]. Clinical studies have also consistently demonstrated that SGLT2 inhibitors protect kidney function by slowing estimated GFR decline and limiting albuminuria in both diabetic and non-diabetic individuals [33]. The benefits of SGLT2 inhibitors go beyond glucose, weight, and blood pressure reduction, which is usually seen with their glucosuric activity in diabetic patients. One of the main mechanisms involved in renal protection is tubuloglomerular feedback, where increased sodium delivery along the nephron is detected by macula densa cells. These cells trigger afferent arteriole constriction through adenosine signaling, thereby lowering intraglomerular pressure and protecting the glomeruli [34,35]. Additional effects of SGLT2 inhibitors include improved tubular oxygenation and metabolism [36], along with reduced renal inflammation and fibrosis [37]. Another renoprotective mechanism of SGLT2 inhibitors is attributed to osmotic diuresis and natriuresis. Upon initiation of SGLT2 inhibitors, the resulting glucosuria leads to osmotic diuresis and transient natriuresis, which diminishes as hyperglycemia improves due to aldosterone compensation [38]. Initial concerns about SGLT2 inhibitors involved risks of genitourinary infections, acute kidney injury, and temporary decline in kidney filtration and blood pressure [39]. However, Bailey et al. revealed that SGLT2 inhibitors have not been linked to a higher risk of urinary tract infections or acute kidney injury [33]. Numerous studies have consistently shown that SGLT2 inhibitors significantly reduce kidney damage, primarily by lowering intraglomerular pressure through normalizing sodium levels and regulating renin and angiotensin II production. As a result, glomerular pressure and hyperfiltration are reduced, protecting the kidneys and correcting neurohormonal disturbances; this leads to alleviation of fluid retention and congestion [40]. In the present study, ERTU alleviated the histopathological damage caused by IFO; this effect might be related to reduced IFO-induced inflammation as evidenced by decreased levels of inflammatory biomarkers derived from CBC: PLR, PMR, and HLR. However, LYCO does not affect these inflammatory biomarkers; this may be attributed to severe leukopenia, which is commonly associated with myelosuppression induced by IFO exposure, which was quite severe to be restored by the short duration of LYCO. Meanwhile, its combination with ERTU resulted in a significant reduction in the value of PLR and reduced PMR and HLR non-significantly. Recent studies have highlighted the value of CBC-derived inflammatory biomarkers such

as PLR, PMR, NMR, and HLR in the early detection, interventions, and management of complications of various diseases like kidney injuries, orthopedics, and cancer [13,41]. The values of PLR, PMR, and HLR showed a significant increase in PC animals in comparison to the NC group. However, their values in rats that used CV, ERTU, and LYCO were comparable with NC groups, with non-significant differences indicating no significant role of these substances in the alteration of these CBC-derived inflammatory biomarkers. Additionally, ERTU reduced oxidative stress status by enhancing renal antioxidant defenses, which was evidenced by elevation of serum level of TAC. This effect is consistent with the findings of a previous study that demonstrated the effect of canagliflozin in reducing oxidative stress through an increase in TAC, superoxide dismutase, catalase, and glutathione reductase activities [11]. In the present study, LYCO attenuated the IFO-induced nephrotoxicity. This was evidenced by a decrease in oxidative stress status, which is represented by a significant elevation in serum TAC. This finding is consistent with the previous studies that demonstrated that LYCO is a strong antioxidant with free radical scavenging activity, and it has been shown to protect kidneys from the deleterious effects of many toxicants and diabetic complications by reducing oxidative stress and improving kidney function markers in animal models [42]. In the present study, the combination of ERTU with LYCO has not increased the net antioxidant capacity; this is inconsistent with previous studies in which LYCO shows a synergistic effect once it is combined with other medications in chemotherapy-induced nephrotoxicity [43]. This discrepancy in the results might be related to the type of nephrotoxicant and the dose and duration of LYCO. ERTU also increased urine output in IFO-treated rats, which may have decreased IFO or its toxic metabolite reabsorption or reduced uptake of IFO in renal proximal tubular cells, thereby contributing to its renoprotective effects [44]. This suggested outcome is supported by the osmotic diuresis and natriuretic feature of SGLT2 inhibition, which may play a more significant role in reno-cardio protection than their ability to lower blood glucose or influence estimated GFR levels [34]. Additionally, in the present study, in IFO-exposed rats, a significant elevation of new protein biomarkers of glomerular function, cystatin C, and tubular injury KIM-1, in the kidney tissue homogenate was observed. This was in agreement with the previous study, which demonstrated renal tubulopathy evidenced by a significant elevation of these protein biomarkers in IFO-induced nephrotoxicity [18]. In the present study, ERTU and its combination with LYCO mitigate cystatin C in IFO-exposed rats significantly. However, LYCO monotherapy decreased it non-significantly. To date, many studies have shown that LYCO supplementation can mitigate kidney damage and inflammation in renal dysfunction by enhancing antioxidant enzyme activity and reducing oxidative stress [15,16]. However, its effect on cystatin C has not been specifically addressed. To the best of our knowledge, the present study is the first to examine the impact of LYCO and ERTU on novel kidney injury biomarkers, including cystatin C. ERTU also mitigates KIM-1 significantly; this effect is aligned with the KIM-1 reduction effect of ERTU in a previous clinical study in which ERTU consistently reduced levels of the KIM-1, irrespective of their initial kidney function in individuals with type 2 diabetes and stage 3 chronic kidney disease [45]. However, LYCO and its combination with ERTU mitigate KIM-1 non-significantly; this finding is inconsistent with a recent study that shows a reduction in serum KIM-1 levels when the animals are pretreated with LYCO in 5-fluorouracil-induced nephrotoxicity [46]. Furthermore, the ameliorative impact of LYCO on cisplatin-induced nephrotoxicity has been documented by Mahmmodnia *et al*. in patients with cancer [42]. Additionally, in another study, a synergistic effect of LYCO has been found once it is combined with the other antioxidant agents, as seen in the combination of LYCO and N-acetylcysteine, which effectively alleviates cisplatin-induced hepatorenal toxicity and apoptosis in rats [47]. This discrepancy may be related to the differences in the samples used for detection of the biomarkers; additionally, the effect of LYCO may be limited and marginal in the presence of massive injury.

Limitations of the study

First, the doses of ERTU and LYCO were chosen based on existing literature without conducting a dose-response analysis. Second, although the study includes macroscopic and microscopic evaluations along with the assessment of novel kidney injury biomarkers, additional biomarkers for kidney injury, such as neutrophil gelatinase-associated lipocalin (NGAL) and clusterin, are recommended for future studies to provide more comprehensive and confirmatory results. Despite these limitations, the study possesses several strengths that support the importance of its outcomes in the clinical field. To the best of our knowledge, this is the first study to evaluate the impact of ERTU and LYCO and their combined protocol in mitigating chemotherapy-induced nephrotoxicity. It lays the basis for future research and highlights the potential nephroprotective role of SGLT2 inhibitors beyond their glucose-lowering properties.

Conclusion

In this study, ERTU and LYCO alone and in their combination alleviated the kidney injury parameters and histopathological lesions induced by IFO significantly. These findings suggest that ERTU and LYCO are effective nephroprotective agents against IFO-induced nephrotoxicity, and their combination added a potential impact on their nephroprotection. The suggested mechanisms for this outcome are attributed to their antioxidant and anti-inflammatory actions for both, as well as diuretic and natriuretic properties for ERTU.

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Conflict of interests

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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