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Assessment of Cyclophosphamide-Induced Nephrotoxicity in a Preclinical Model and Evaluation of the Protective Efficacy of Sylvestroside I via Modulation of Pro-Inflammatory Cytokine Pathways

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ABSTRACT

Cyclophosphamide (CYP) is a potent chemotherapeutic and immunosuppressive agent whose clinical utility is limited by dose-dependent nephrotoxicity which its toxic metabolite, acrolein, triggers oxidative stress, inflammatory cytokine imbalance, and histopathological damage in the kidney. At last years a different study on natural compounds with antioxidant and anti-inflammatory properties are increasingly investigated for their potential to mitigate CYP-induced renal injury since sylvestroside I (SYL), a secoiridoid glycoside, has been suggested to possess protective effects, but its role in nephroprotection remains unexplored. The present study aimed to evaluate the protective role of Sylvestroside I against the CYP that induced nephritis in rats through assessment of renal histopathology, oxidative stress, and inflammatory cytokine included Interleukin-6 Interleukin-22 Tumor Necrosis Factor-Alpha. Forty adult albinos male Wistar rats weighing between 150 to 200g were divided randomly into four groups, with 10 rats in each group. The groups included Group I (CON) received vehicle only while the Group II (CYP) received a single intraperitoneal injection of CYP (200 mg/kg) on the first day of the study only on other hands the Group III (SYL) administered Sylvestroside I orally at a dosage of 50 mg/kg/day. The Group IV (CYP+SYL) received both CYP (200 mg/kg, i.p.) and SYL (50 mg/kg/day orally). After 30 days of experimental at the end of study a biochemical marker including serum creatinine, blood urea nitrogen (BUN), malondialdehyde (MDA), and total antioxidant capacity (TAC) were analyzed in additionally to Cytokines TNF-α, IL-6, and IL-22 and Kidney tissues were examined histopathologically. CYP administration induced significant nephrotoxicity, evidenced by elevated serum creatinine, BUN, TNF-α, and IL-6, along with reduced TAC and IL-22 as well as th histopathological evaluation showed glomerular shrinkage, tubular necrosis, and interstitial inflammation. the grope of SYL alone that showed maintained the renal in normal architecture and biochemical balance. Notably, SYL as a co treatment significantly against CYP-induced alterations, restoring IL-22, lowering TNF-α and IL-6, reducing oxidative stress, and preserving renal tissue integrity. Sylvestroside I exhibit as nephroprotective effects against CYP-induced nephritis through antioxidant activity and modulation of inflammatory cytokines which these findings highlight its potential as a therapeutic adjunct to mitigate chemotherapy-related renal injury, warranting further mechanistic and clinical investigation.

1-Introduction

Cyclophosphamide is a nitrogen mustard alkylating agent, which has been used clinically widely to provide treatment for malignant diseases, such as lymphomas, breast cancer, and leukemias, moreover used for autoimmune diseases as systemic lupus erythematosus and rheumatoid arthritis. While the therapeutical application of cyclophosphamide is wide, the clinical utility has been decided in a big way by the dose-limiting toxicities, and nephrotoxicity has been evidently at the forefront [1,2]. Kidneys are very sensitive to xenobiotic-triggered damage because these organs are involved in purification of the blood and concentrating metabolites that are toxic. Furthermore, the cyclophosphamide undergoes metabolism during the metabolic process, it metabolizes to the active species phosphoramide mustard, the molecule responsible for cytotoxicity, and the very reactive metabolite acrolein that triggers oxidative stress, lipid peroxidation, and DNA lesions in renal tissue [3,4]. The pathogenesis of cyclophosphamide nephrotoxicity involves a multi-factorial process involving oxidative stress, inflammation, and apoptosis. Experimental studies have found that cyclophosphamide induction leading to a rise in serum creatinine and blood urea nitrogen to be consistent with impaired glomerular filtration and renal impairment [5,6,7].

Inflammatory cytokines are no less important in cyclophosphamide induced renal lesions. TNF-α is one of such pro-inflammatory cytokines to initiate the down-stream signaling cascades to apoptosis and necrosis of tubular cells in the kidneys [8,9,4]. Similarly, IL-6 has been found to have a role in the facilitation of the enhancement in inflammation and the induction in leukocyte recruitment to renal parenchyma, thus leading to tubular degeneration and glomerular atrophy [10,11,12]. On the other hand, IL-22 has been found to have a protective one as a cytokine by initiating the induction in the signaling pathway JAK2/STAT3 to induce cell survival, repair, and regeneration in the model of nephritis caused by cyclophosphamide [13]. The interaction among the above cytokines thus determines the direction in tissue destruction or repair in the model of nephritis caused by cyclophosphamide.

There have been recent efforts to investigate the activity of the natural antioxidants and plant molecules as adjuvant therapies to combat cyclophosphamide nephrotoxicity. The phytoconstituents berberine, thymoquinone, and phenolic fractions have been found to show nephroprotection by down-regulation of oxidative stress and modulation of pro-inflammatory cytokines and thus mediate histological and functional repair in animal models [14,15,16]. Sylvestroside I is a plant-derived secoiridoid glycoside reported in medicinal botanical species and recognized for antioxidant and anti-inflammatory bioactivity. Emerging evidence suggests that structurally related secoiridoids exert renoprotective effects through radical scavenging and modulation of inflammatory signaling pathways [3].

Therefore, the present study was designed to evaluate the histopathological and biochemical alterations associated with cyclophosphamide induced nephritis and to investigate the renoprotective efficacy of Sylvestroside I.

2. Material and methods

Ethics Approval:

All procedures and animals was approved by the guide line of Institutional Animal Care and Use Committee of The Islamic University, Najaf (2025-2-32; 432Is), that following the ethical guidelines for laboratory animal research.

Animals

The forty healthy male albino Wistar rats that was weighing between 150 and 200 g was used in the present study experiment additionally the animals were obtained from the Abu grab, Baghdad, Iraq. They were housed in standard international cages at the Animal House at Najaf, under controlled environmental conditions that temperature 25–30 °C furthermore, all rats were maintained on a standard pellet diet with free access to water throughout the study period.

Study design

The experiment was used 40 healthy adults male Wistar rats and randomly divided into four equal groups (n = 10 each) and observed for 30 days which the Group I (CON) received a vehicle only and served as the control reference. Group II (CYP) the Cyclophosphamide was administered a single intraperitoneal dose of cyclophosphamide (200 mg/kg) on the first day of the experiment to induce nephrotoxicity [1,17]. Group III (SYL) received Sylvestroside I alone (50 mg/kg/day orally) to evaluate its independent effects. Cyclophosphamide (200 mg/kg, intraperitoneally) and Sylvestroside I (50 mg/kg/day orally) were supplied to Group IV (CYP+SYL) to assess the protective efficacy of the compound. At the end of study protocol after 30 days of experimental the blood samples were collected at the end of the thirty-day treatment period to perform a biochemical analysis of serum creatinine, blood urea nitrogen (BUN), total antioxidant capacity (TAC), malondialdehyde (MDA), and the investigation of cytokines, TNF-α, IL-6, and IL-22 furthermore kidney tissues were obtained for histological analysis.

Cyclophosphamide

Cyclophosphamide was obtained from Baxter Healthcare Corporation (Deerfield, IL, USA; Germany) and supplied locally through the Al-Faiha Company in Najaf, Iraq and the drug was prepared and handled in accordance with the manufacturer's instructions.

Sylvestroside I

Sylvestroside I (Cat. No. HY-N3030, purity 98%, CAS No. 71431-22-6) was obtained in purified form from MedChemExpress, USA (Figure 1). For experimental administration, Sylvestroside I was freshly suspended in 0.5% of carboxymethylcellulose aqueous solution, which served as the vehicle and ensured oral. The suspension was prepared daily to maintain stability and prevent compound degradation and administered immediately after preparation moreover the rats in the treatment groups received Sylvestroside I orally at a dose of 50 mg/kg/day for 30 consecutive days.

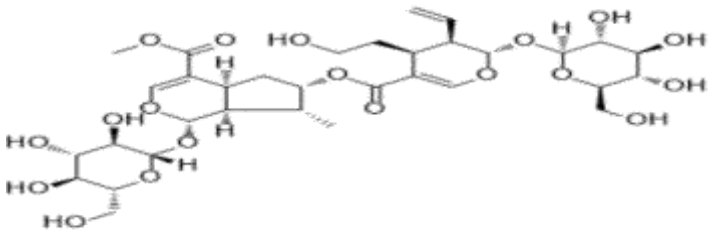

Figure 1. Chemical structure of Sylvestroside I

Interleukin-6 (IL-6)

The IL-6 concentrations was evaluation by using a rat Interleukin-6 (IL-6) ELISA kit (SunLong Biotech Co., Ltd., China; Cat. No. SL0411Ra) which this intended for sensitive detection of IL-6 in rat serum, which the sample preparation was performed according to the manufacturer's instructions since the serum samples were obtained after clotting and centrifugation, standards, controls, and prepared samples were added to antibody-coated wells, followed by incubation with an HRP-conjugated IL-6 antibody. After thorough washing, chromogen solutions A and B were introduced to develop color, and the reaction was terminated with stop solution. Absorbance was measured at 450 nm using a microplate reader, and IL-6 concentrations were calculated from a standard curve generated with known calibrators.

Interleukin-22 (IL-22)

The IL-22 levels were measured using a commercial Rat Interleukin-22 (IL-22) ELISA kit (SunLong Biotech Co., Ltd., China; Cat. No. SL0790Ra) which this intended for sensitive detection of IL-22 in rat serum, which the sample preparation was performed according to the manufacturer's instructions since the serum samples were obtained after clotting and centrifugation, standards, controls, and prepared samples were added to antibody-coated wells, followed by incubation with an HRP-conjugated IL-22 antibody. After thorough washing, chromogen solutions A and B were introduced to develop color, and the reaction was terminated with stop solution. Absorbance was measured at 450 nm using a microplate reader, and IL-22 concentrations were calculated from a standard curve generated with known calibrators

Tumor Necrosis Factor-Alpha (TNF-α)
To evaluate the TNF-α concentration using a ELISA kit (Melsin, China; Cat. No. EKRAT-0419) per manufacturer's instructions which after serum sample collection that was incubated with particular detection antibodies and enzyme conjugates in antibody coated wells and after adding the substrate solution, a microplate reader measured colorimetric development at the appropriate wavelength, finally TNF-α concentrations were measured using a standard curve based on pre-determined cytokine levels.

Total Antioxidant Capacity (TAC)

Total antioxidant capacity (TAC) was determined using the T-AOC Assay Kit (Solarbio Life Sciences, China; Cat. No. BC1315) scince the serum was separated from whole blood by centrifugation at 3000 × g for 10 min at 4 °C, aliquoted, and stored at −80 °C until analysis then a samples were prepared using an extraction solution precooled to 2–8 °C. The standard solution (40 μmol/mL) was prepared by dissolving 10 mg of the standard powder in 0.9 mL distilled water with 20 μL concentrated H_2SO_4. A working reaction mixture was freshly prepared by mixing Reagents I, II, and III in a 7:1:1 ratio and the serum samples and standards were added to the reaction mixture in microplate wells, and after incubation and centrifugation, the absorbance of the supernatant was measured at 593 nm using a spectrophotometer.

Malondialdehyde (MDA)

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) levels in serum using the MDA Content Assay Kit (Solarbio Life Sciences, China; Cat. No. BC0025). Blood samples were allowed to clot at room temperature and centrifuged at 3000 × g for 10 min to obtain clear serum. The serum samples were mixed with the MDA working reagent and Reagent III than mixture was incubated at 100 °C for 60 min to allow condensation of MDA with thiobarbituric acid under acidic conditions and after cooling and

centrifugation, the absorbance of the supernatant was recorded at 532 nm using a spectrophotometer.

Histopathological examination of kidneys

For histopathological assessment, the kidneys were carefully excised, longitudinally opened, and immediately fixed in 10% neutral-buffered formalin for 48 hours at room temperature (22–25 °C). The fixed tissues were then processed following standard histological procedures, which included graded dehydration through ascending concentrations of ethanol, clearing in xylene (two stages), and embedding in paraffin wax at 56 °C for approximately 2 hours. The obtained sections were dewaxed, rehydrated, and stained with Harris hematoxylin and eosin (H&E). Finally, the stained slides were examined under a light microscope at magnifications of 4×, 10×, and 40× to evaluate renal histoarchitecture and pathological alterations.

Statistical Analysis

All data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) whic the Shapiro–Wilk test was applied to assess the normality of data distribution. The differences among experimental groups were evaluated using a mixed-model, repeated-measures one-way analysis of variance (ANOVA) and a p-value of less than 0.05 was considered statistically significant.

3.Result

The Serum levels of TNF-α were significantly altered among the experimental groups (Figure 2) which the CYP group exhibited a marked elevation in TNF-α concentration at (104.56 ng/L) compared to the control group (CON, 60.20 ng/L; at $p < 0.0001$). On other hand administration of SYL I alone did not produce significant change compared with the control (62.76 ng/L; ns), that indicates its lack of pro-inflammatory effect. Notably, cotreatment with Sylvestroside I CYP+SYL significantly reduced TNF-α levels (50.79 ng/L) compared with the CYP group at $p < 0.0001$, bringing the values closer to normal and even slightly lower than the control group at $p < 0.01$. The results of this study provide evidence that cyclophosphamide is responsible for inducing a robust pro-inflammatory response by means of the overexpression of TNF-α. On the other hand, Sylvestroside I is able to effectively reduce this surge of cytokines and suggesting a beneficial anti-inflammatory characteristic.

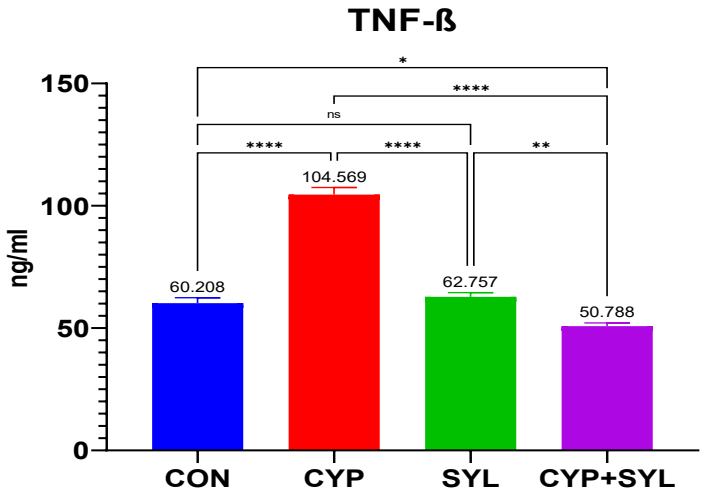

Figure 2. Serum TNF-α levels in experimental groups. Cyclophosphamide administration (CYP) significantly increased TNF-α compared to the control (CON). Sylvestroside I alone (SYL) did not alter

TNF-α levels compared with the control, whereas co-treatment (CYP+SYL) markedly reduced TNF-α compared to CYP, demonstrating an anti-inflammatory protective effect. Data are expressed as mean ± SEM; ns = non-significant, **p < 0.01, ****p < 0.0001.

As shown in the Figure 3, the serum of IL-6 levels evalouted significantly among the experimental groups while the cyclophosphamide administration led to a significant increase in IL-6 concentration 195.20 ng/ml compared to the control group (CON, 175.59 ng/L; at p < 0.0001) on other hand the treatment with sylvestroside I alone (178.86 ng/L) showed no significant difference compared with the control (ns), indicating that Sylvestroside I did not induce an inflammatory response when given independently. In contrast, co administration of Sylvestroside I with cyclophosphamide CYP+SYL showed the significantly reduced IL-6 levels (163.37 ng/L) compared to the CYP group at p < 0.0001 and even showed a modest reduction compared with the control group at p < 0.01.

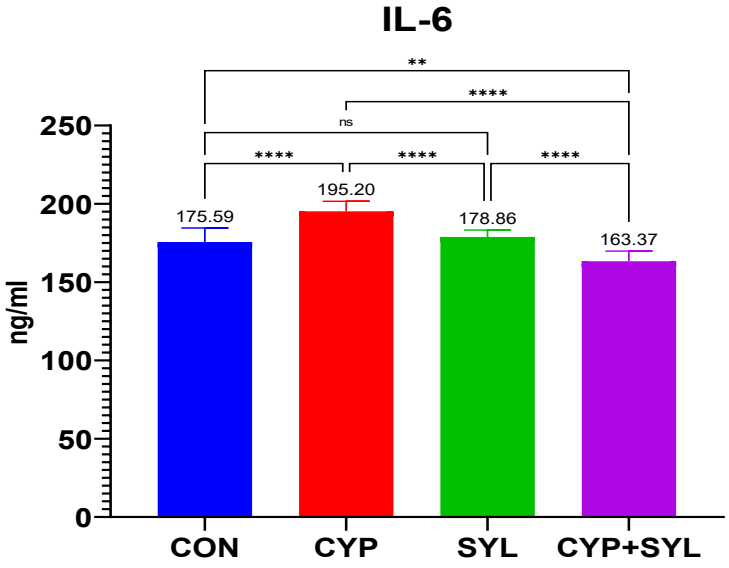

Figure 3. Serum IL-6 levels in experimental groups. Cyclophosphamide (CYP) induced a significant elevation in IL-6 compared with control (CON). Sylvestroside I alone (SYL) showed no significant difference from control, while co-treatment (CYP+SYL) significantly reduced IL-6 compared with CYP, indicating attenuation of inflammatory response. Data are expressed as mean ± SEM; ns = non-significant, **p < 0.01, ****p < 0.0001.

As illustrated in Figure 4, IL-22 levels were significantly altered by cyclophosphamide treatment and Sylvestroside I administration. The control group (CON) exhibited baseline IL-22 concentrations (46.89 ng/ml). Cyclophosphamide administration (CYP) caused a pronounced reduction (23.07 ng/L; ****p < 0.0001 vs. CON), indicating suppression of this protective cytokine. Sylvestroside I alone (SYL) partially restored IL-22 levels (35.45 ng/L; ****p < 0.0001 vs. CYP, but still significantly lower than CON). Interestingly, the co-treatment group (CYP+SYL) demonstrated a substantial elevation (48.56 ng/L), which was significantly higher than the CYP group (****p < 0.0001) and comparable to the control group (ns). These findings suggest that Sylvestroside I not only mitigated the CYP-induced suppression of IL-22 but also restored its levels to near-normal, highlighting its role in promoting immune protection and tissue repair.

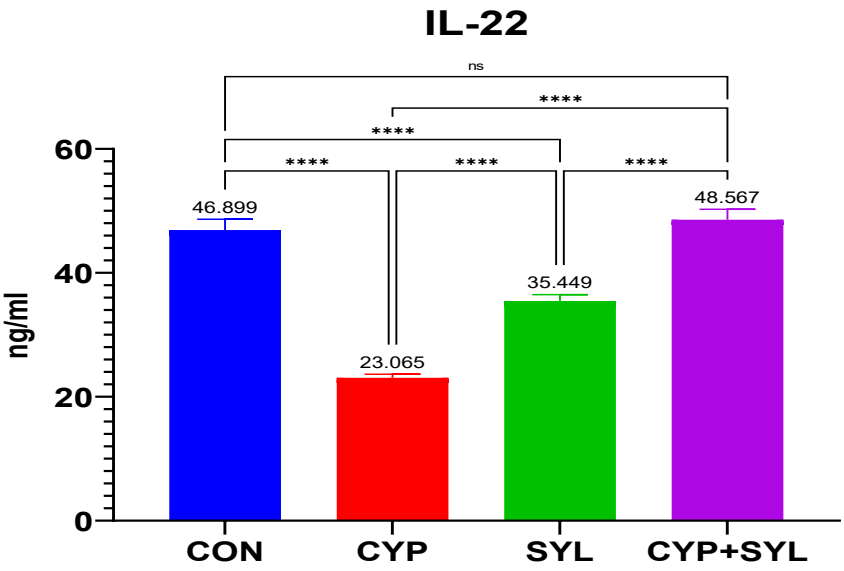

Figure 4. Serum IL-22 levels in experimental groups.
Cyclophosphamide administration (CYP) significantly reduced IL-22 levels compared with the control group (CON). Sylvestroside I treatment (SYL) maintained IL-22 values comparable to control, while co-treatment (CYP+SYL) significantly restored IL-22 levels relative to CYP, indicating a modulatory protective effect of Sylvestroside I. Data are expressed as mean ± SEM; statistical significance indicated as ns = non-significant, **p < 0.01, ****p < 0.0001.

The Blood MDA levels in Figure 5 show that there was not significant differences among the experimental groups which the CON group showed a value at 1.55 nmol/mL, while the CYP group decreased but nonsignificant decreased to (1.25 nmol/mL) in other hand the rats was treated with SYL alone showed levels similar to the control at 1.50 nmol/mL, indicating no notable oxidative imbalance. Interestingly, the cotreatment group (CYP+SYL) shwed a mild elevation in MDA (1.71 nmol/mL), yet this change was also nonsignificant compared to both the control and CYP groups.

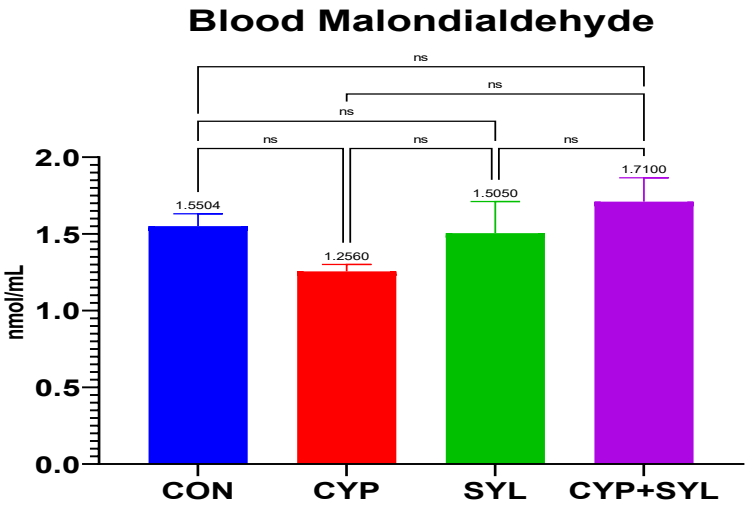

Figure 5. Blood malondialdehyde (MDA) levels in experimental groups. No significant differences were observed in MDA levels among the control (CON), cyclophosphamide (CYP), Sylvestroside I (SYL), and co-treatment (CYP+SYL) groups, indicating that neither CYP nor Sylvestroside I markedly influenced systemic lipid peroxidation under these conditions. Data are expressed as mean ± SEM; ns = non-significant.

The figure 6 illustrated a total antioxidant capacity levels varied significantly across the groups which the CON group exhibited the highest TAC value (7.35 nmol/mL), while CYP group resulted that significant decline at (6.48 nmol/mL; $p < 0.0001$ vs. CON) on other hand the SYL group showed a marked reduction in TAC (2.24 nmol/mL; $p < 0.0001$ vs. CON and CYP), similarly, the co-treatment group (CYP+SYL) displayed low TAC levels (2.48 nmol/mL), which were significantly reduced compared with both the control and CYP groups ($p < 0.001$). No significant difference was detected between SYL and CYP+SYL groups.

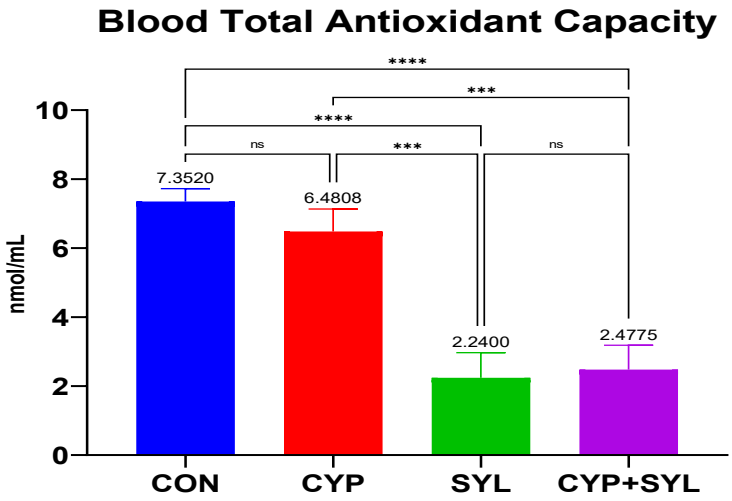

Figure 6. Blood total antioxidant capacity (TAC) in experimental groups. Control rats (CON) exhibited the highest TAC values. Cyclophosphamide (CYP) significantly reduced TAC compared with control. Both Sylvestroside I alone (SYL) and co-treatment (CYP+SYL) showed further significant reductions in TAC compared with CON and CYP, with no significant difference between SYL and CYP+SYL. Data are expressed as mean ± SEM; ns = non-significant, ***$p < 0.001$, ****$p < 0.0001$.

Histological examination of renal tissue from the control group (Figure 7-A) revealed a normal kidney architecture which the glomerulus appeared intact, with well-defined capillary tufts and normal mesangial cellularity and bowman's capsule was preserved, showing no signs of thickening or distortion as well as the surrounding renal tubes maintained their typical morphology with no evidence of degeneration, necrosis, or inflammatory infiltration. The renal sections from the cyclophosphamide treated group showed (Figure 7-B) marked histopathological alterations compared to the control which the glomeruli exhibited shrinkage with widened Bowman's space and partial loss of normal architecture. Additionally, the tubular structures displayed vacuolar degeneration, cytoplasmic swelling, and focal necrosis. Moreover, Inflammatory cell infiltration was also observed in the interstitial areas. Kidney tissue from rats treated with Sylvestroside I alone demonstrated largely preserved renal histoarchitecture which glomeruli appeared shwed normal with intact capillary tufts and no apparent thickening of the Bowman's capsule, additionally mesangial cells were within normal limits, and the surrounding renal tubules maintained their typical structure with only minimal alterations. These findings indicate that Sylvestroside I, when administered alone, does not exert nephrotoxic effects and maintains normal renal morphology (Figure 7-C). Renal sections from rats co-treated with cyclophosphamide and Sylvestroside I (Figure 7-D) showed a marked improvement in kidney architecture compared to the cyclophosphamide group alone which the glomeruli appeared more preserved, with nearly normal capillary tufts and reduced mesangial proliferation

additionally the Bowman's space was less dilated, and tubular structures showed minimal degenerative changes.

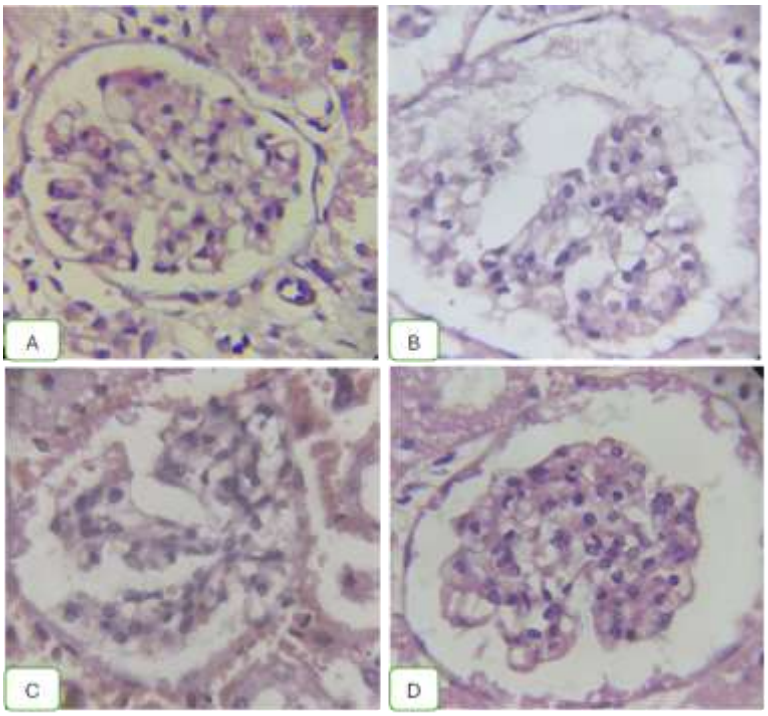

Figure 7. Histopathological changes in kidney tissues of experimental groups (H&E staining, ×400). (A) **Control group (CON):** Normal renal histology with intact glomerular architecture, preserved Bowman's capsule, and well-organized renal tubules. (B) **Cyclophosphamide group (CYP):** Marked pathological alterations, including glomerular shrinkage, widened Bowman's space, tubular degeneration, and inflammatory infiltration, indicating nephrotoxicity. (C) **Sylvestroside I group (SYL):** Preserved kidney structure with intact glomeruli and tubules, showing no significant histological abnormalities. (D) **Cyclophosphamide + Sylvestroside I group (CYP+SYL):** Improved renal architecture with near-normal glomeruli, reduced tubular degeneration, and attenuated inflammatory changes, demonstrating the protective effect of Sylvestroside I against cyclophosphamide-induced injury.

4.Discussion

The present study showed that Sylvestroside I co-treatment was associated with improvements in oxidative stress markers, antioxidant defenses, and cytokine profiles in a cyclophosphamide-induced nephrotoxicity model. Our results revealed that CYP administration led to marked renal injury characterized by histopathological damage, elevated serum creatinine and BUN, increased MDA, reduced TAC, and dysregulation of cytokines, notably elevated TNF-α and IL-6 with concomitant suppression of IL-22. Co-treatment with SYL significantly reversed these alterations, supporting its potential as a nephroprotective compound [18].

As suggested elsewhere [3,14], the present data show that phytochemical-mediated modulation of oxidative stress pathways contributes to renal protection. The CYP nephrotoxicity is primarily mediated through its toxic metabolite acrolein, which induces reactive oxygen species (ROS) generation, lipid peroxidation, and activation of pro-inflammatory cascades [3,19]. Consistent with our findings, several studies have reported increases in oxidative stress markers such as MDA and decreases in antioxidant enzymes (SOD, CAT, GSH) following CYP exposure [8,9,20]. The observed rise in TNF-α and IL-6 in the CYP group aligns with evidence that these cytokines are central mediators of CYP-induced renal inflammation via NF-κB and MAPK activation [14,21]. Interestingly, IL-

22, a cytokine with renoprotective roles in tissue repair and inflammation resolution, was markedly suppressed in the CYP group, echoing reports that nephrotoxins attenuate IL-22 signaling and thereby compromise renal recovery [22,13]. Restoration of IL-22 by SYL indicates a potential role in balancing pro- and anti-inflammatory cytokine responses. These findings raise the possibility that SYL may contribute to regenerative pathways, similar to effects reported with other phytochemicals [23,4] and formononetin [14,24]. The antioxidant potential of SYL, as evidenced by reduced MDA and improved TAC, is consistent with studies showing that natural iridoid glycosides and polyphenolic compounds counteract CYP-induced redox imbalance [3,25]. Similar nephroprotection was observed with synaptic acid [26,19], thymoquinone [27], and cinnamaldehyde [28,29], all of which enhance Nrf2-mediated antioxidant responses and attenuate NF-κB-driven inflammation.

Our histological findings further support the biochemical results, where CYP caused glomerular shrinkage, tubular necrosis, and interstitial infiltration, in line with previous reports of CYP-induced nephropathy [3,30]. SYL administration mitigated these alterations, paralleling the effects of other phytochemicals such as berberine [4] and garlic extract [31,32].

Based on the observed biochemical and cytokine changes, SYL act through dual mechanisms: reducing oxidative stress and inflammatory mediators (e.g., TNF-α, IL-6), and enhancing protective cytokines such as IL-22. While our data cannot confirm signaling pathway involvement, these patterns are consistent with previous reports of phytochemicals acting on NF-κB, MAPK, and Nrf2 pathways [25], and (ii) upregulation of protective cytokines like IL-22, which promotes renal epithelial regeneration and dampens chronic inflammation [33,13]. This mechanistic profile aligns with other nephroprotective compounds that target the Nrf2/HO-1 and NF-κB/NLRP3 inflammasome pathways [3,2].

Taking together, our findings suggest that phytochemicals such as SYL may mitigate CYP-induced toxicity by improving redox balance and modulating immune responses. These results warrant further mechanistic studies to clarify the pathways involved and to explore potential translational applications.

5.Conclusion

The present study demonstrated that cyclophosphamide induces profound nephrotoxicity characterized by oxidative stress, inflammatory cytokine imbalance, and histopathological alterations. Co-treatment with Sylvestroside I was associated with attenuation of these changes, including lower TNF-α and IL-6 levels, partial restoration of IL-22, improved antioxidant capacity, and better preservation of renal architecture. These findings suggest that Sylvestroside I may confer nephroprotective potential, possibly through modulation of oxidative stress and inflammatory pathways, although further mechanistic studies are required to confirm these effects.

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