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Advancing Renal Health in Sepsis Management: The Promising Role of Thymoquinone through PI3K/Akt Signaling Modulation

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Abstract

Acute Kidney Injury (AKI), a major health challenge, is predominantly triggered by sepsis. Thymoquinone (TQ), extracted from *Nigella sativa*, that is recognized for its potent antiinflammatory in addition to antioxidant capabilities. This study provides strong evidence for the efficacy of Thymoquinone (TQ) in attenuating sepsis-induced Acute Kidney Injury (AKI), highlighting its potential as a therapeutic agent through modulation of inflammation and oxidative stress via PI3K/Akt signaling.

The experiment allocated twenty-five albino mice into four groups (n=5 each): (1) Sham group (underwent laparotomy alone without CLP), (2) Sepsis control group (administered CLP alone without treatment), (3) TQ-treated group (treated with 0.75 mg/kg thymoquinone intraperitoneally for three consecutive days prior to CLP), and (4) Vehicle group (administered normal saline for three consecutive days prior to CLP). Except for the sham group, all the animals were subjected to the CLP procedure to induce sepsis. After the procedure, a set of biochemical, immunohistochemical, and histopathological tests were performed to evaluate renal status and inflammatory milieu. These indicated that treatment with TQ substantially halted serum levels of proinflammatory mediators IL-6 and TNF-α but higher levels of antiinflammatory cytokine IL-10. Besides that, levels of creatinine and urea were reduced, reflecting improved kidney function. Histopathological analysis revealed that TQ exerted protective effect against CLP-induced nephropathy. On the level of cell signaling, the modulating impact of TQ on the PI3K/Akt pathway was reflected by increased pAkt immunoreactivity and elevated PI3K gene expression.

This research highlights the potential of TQ in attenuating sepsis-induced AKI, mediated through modulation of inflammation, oxidative stress, and activation of the PI3K/Akt pathway

Keywords*:* Sepsis; Thymoquinone; *Nigella Sativa;* Acute Kidney Injury; PI3K/Akt Pathway; Inflammation; Cytokines; Oxidative Stress

1. Introduction

Phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway is the one that controls a broad spectrum of cellular functions key to the pathogenesis of sepsis. They encompass regulation of the immune responses and release of inflammatory mediators in vitro and in vivo (Geng *et al.*, 2024) (Al-Husein *et al.*, 2020). Activation of the pathway influences downstream signaling molecules. It is essential in the modulation of innate immune cell functions, playing a part in immune regulation and the preservation of homeostasis during sepsis. The complication of the PI3K/Akt/mTOR signaling components emphasizes its complex function in cellular signal transduction, influencing cell proliferation,

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apoptosis, metabolism, and angiogenesis. These features emphasize the pathway's critical role in numerous human diseases, where its dysregulation can intensify conditions as ischaemic brain injury, neurodegenerative diseases, in addition to cancers (Bi *et al.,* 2023). In sepsis, studying the PI3K/Akt pathway's governing mechanisms and biological functions offers promising avenues for the progress of targeted therapies to control immune and inflammatory responses, theoretically preventing the critical outcomes of sepsis induced Acute Kidny Injury (AKI) (Li *et al.,* 2023). Sepsis is a significant global health issue. It induces a robust inflammatory response that can cause systemic dysregulation and multiorgan failure (Trzeciak *et al.,* 2020). The kidneys, mainly vulnerable in sepsis, suffer from 45% to 70% of ICU septic patients evolving Acute Kidney Injury (AKI) (Chang *et al.,* 2022) (Abu Saleem *et al.,* 2025).

Existing evidence suggests that AKI in sepsis is an active performer of the inflammation tissue injury flow and not a passive result of systemic inflammatory processes (Jarczak *et al.,* 2021; Ludes *et al.,* 2021). Central to such understanding lies the Phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway significant for cell growth, survival, and progressively being included in the pathogenesis of sepsis-related AKI (Peerapornratana *et al.,* 2019; Ludes *et al.,* 2021).

With the growing understanding of sepsis and AKI pathophysiology, thymoquinone (TQ), the principal bioactive constituent of Nigella sativa, has attracted increasing attention because of its diverse pharmacological properties, including anti-inflammatory, antioxidant, immunomodulatory, and anti-apoptotic effects (Talebi *et al.*, 2021) (Al-Saffar and Al-Wiswasy, 2019). Thymoquinone was selected due to its unique blend of anti-inflammatory, antioxidant, and immunomodulatory action, coupled with a well-documented safety record in preclinical models. (Isaev *et al.*, 2023) Unlike the conventional anti-sepsis medications that focus primarily on single pathways, TQ has pleiotropic effects, which make it exceptionally potent for multifactorial conditions such as sepsis-induced AKI.(Farkhondeh *et al.*, 2018; Pottoo *et al.*, 2022). Research in disease models implies TQ's ability to reduce inflammatory markers and diminish tissue damage (Ojha *et al.*, 2015) (Alqaraleh *et al.*, 2025), mainly through modulation of PI3K/Akt signaling, as noticed in LPS-induced neurotoxicity in microglial cells and renoprotection with cisplatin induced nephrotoxicity (Ojha *et al.*, 2015) (Sayed and Morcos, 2007).

Despite massive research, mechanisms supporting sepsis and sepsis-related AKI remain unclear, leading to a nonstop search for novel, effective therapies. TQ's preclinical success in modulating vital cellular processes across the PI3K/Akt axis—crucial in diseases like sepsis and AKI — emphasizes its therapeutic potential (Junaid *et al.,* 2021). These characteristics render TQ as a candidate drug for sepsis-induced AKI, the condition characterized by uncontrolled inflammation and oxidative renal damage. Recent findings suggest that TQ reduces kidney damage in various models of nephrotoxicity and ischemia-reperfusion injury (Wang *et al.*, 2022). The specific role of TQ in regulating the PI3K/Akt signaling pathway in sepsis-induced AKI has not been explored before, which is the focus of the present study. By its blockage of this critical pathway, TQ may provide a novel therapeutic approach to sepsis-induced nephrotoxicity

As illustrated by Öztürk et al. and Qadri et al., the gentamicin kidney damage model, the AKI model of cisplatin, and LPS-driven lung injury suggest that TQ has therapeutic potential for managing sepsis.(Öztürk *et al.,* 2023; Qadri *et al.,* 2023). Furthermore; Li-Peng Guo et al. results indicated that TQ might have a potential therapeutic activity addressing sepsis-induced AKI(Guo *et al.*, 2020). Consequently, our current activities focus on investigating the proposed PI3K/Akt pathway and TQ's renoprotective actions against polymicrobial sepsis. Based upon TQ's established renoprotective features, we aim to elucidate the therapeutic potential of TQ in the context of sepsis and AKI, which would signify a remarkable treatment milestone.

2. Materials & Methods

2.1. Chemical Reagents and Suppliers

Thymoquinone (TQ) source was Sigma Aldrich (USA). Enzyme linked Immunosorbent Assay (ELISA) kits for the examination of Interleukin-6 (IL-6), Tumor Necrosis Factor-alpha (TNF-α), TNF-α receptor, Interleukin-10 (IL-10), and F2-isoprostanes were provided by SunLog Biotech (China). Solarbio (China) supplied the Total RNA extraction kit. Biotinylated secondary antibody reagent, antigen retrieval buffer, Syper green PCR master mix, and blocking buffer were sourced from Thermo Fisher Scientific (UK). Protease cocktail inhibitor tablets were procured from Roche (Germany). Hikma (Jordan) and Bayer AG (Germany) provided Ketamine and Xylazine, respectively.

2.2. Animal Preparation and Ethical Approval

A total of twenty-five albino mice, ranging in age from 8 to 12 weeks and in weight from 20 to 30 gram, were obtained from the Animal Care Centre within the Faculty of Science at the University of Kufa, Iraq. These mice were then housed in an environmental system regulated for temperature (25° Celsius) and humidity (60-65%), with a consistent 12:12 light/dark sequence. All mice had free access to foodstuff and water. The experimental processes were reviewed and approved by the "Animal Care and Use" Committee at the University of Kufa, Iraq, under license number 15791, ensuring adherence to ethical standards.

2.3. Experimental Design

Following acclimatization, mice were allotted to groups at random in a manner of five mice per group. The groups were: sham group, where the mice were anesthetized and a midline abdominal incision made to simulate surgery; control group, where the mice were anesthetized and induced with Cecal Ligation and Puncture (CLP); TQ-treated group, treated with intraperitoneal (i.p) administration of Thymoquinone (TQ) in a dose of 0.75mg/Kg/day for three days prior to the CLP procedure (Hiengrach *et al.*, 2022), normal saline-treated group, treated with i.p injections of normal saline employed as a control vehicle for TQ, and administered for three days prior to the induction of CLP. The normal saline-treated group, which received i.p. saline for three consecutive

days was then subjected to CLP to induce sepsis, similar to the control and TQ-treated groups

2.4. Dose Selection

TQ dose was 0.75mg/kg/day, and it was selected based on our prior studies showing its efficiency and safety in comparable experimental models (Alkharfy *et al.*, 2011; Alkharfy *et al.*, 2015). It was therapeutically efficient without side effects in models of systemic inflammation and sepsis, in harmony with the objectives of the existing study to inspect the protective outcomes of TQ against sepsis induced renal damage.

2.5. CLP Procedure

CLP model was accomplished with caution following standard protocol (Zhai *et al.*, 2018; Guo *et al.*, 2020). Starting with, under humane concern, mice were anesthetized by intraperitoneal injection, through a regulated amount of 100mg/kg Ketamine and 10mg/kg Xylazine. Once anesthetized, strict preparation like shaving the abdominal region and making a 1.5 cm midline incision to expose the cecum. This vital organ was gently shown, carefully ligated below the ileocecal valve to prevent leakage, and punctured twice with a sterile G-20 needle, the infusion of a precise injury planned to cause severe sepsis. Anatomical integrity preserved, the cecum was then replaced within its original position in the abdominal cavity. Procedure was settled with closure of the incision, reasserting commitment to ideal standards of surgery and research integrity.

2.6. Survival Data

Survival of mice was observed for 24 hours following the CLP procedure. Regular monitoring time intervals of four hours was adopted to capture any change in the subjects' condition so as to get appropriate post-operative care. This long period of observation was required to assess the immediate effect of CLP-induced sepsis on survival.

2.7. Sample Collection

Mice were sacrificed and samples were taken 24 hours after the CLP procedure. Blood volume of 0.5-1 ml was drawn directly from the heart, and kidneys were removed. One of the kidneys was stored in a 10% formalin solution, and the other was stored with storage at a temperature of -80C°.

2.8. Biochemical Analysis

Room temperature blood samples were allowed to clot for 30 minutes in order for serum to separate. Samples were then centrifuged, after clotting at the force of 1500 xg for a period of 10 minutes. The single serum was then assayed to establish levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), TNF-α receptor, and interleukin-10 (IL-10) via enzyme-linked immunosorbent assay (ELISA) kits from Sunlong Biotech Co., Ltd. All the processes in the experiments were done strictly to the letter of the manufacturer's guidelines to determine accuracy and reliability of the results. In addition, blood urea and creatinine contents were estimated by using diagnostic reagents provided by Roche and providing the integral picture information for kidney function in the context of biochemical investigations.

2.9. Histological Examination

Fixed kidney samples in formalin were histologically processed by paraffin embedding, sectioning into sections of 5 µm thickness, and subsequently deparaffinized and stained with Hematoxylin and Eosin (H&E) (Shraideh *et al.*, 2013). Stained tissue sections under a light microscope were evaluated by three different examiners.

2.10. Immunohistochemical Study

Immunohistochemistry analysis was performed according to instructions provided in the Biotin-Labeled Secondary Antibodies kit protocol at Thermo Fisher Scientific, UK. Formalin-fixed paraffin-embedded sections of kidneys were deparaffinized by two washes for ten minutes in xylene. Sections were then hydrated in a graded series of ethanol and three times phosphate-buffered saline wash. 1:100 diluted pAkt antibody was applied to the sections and left for overnight incubation at 4C°. The sections were washed three times in PBS after incubation. The sections were then treated with a secondary antibody conjugated to the horseradish peroxidase enzyme and incubated for a sufficient time at 37℃. As a step toward the development of the stain, a DAB solution was brought into contact with the sections followed by a hematoxylin two-minute counterstain. The sections were then dehydrated with xylene and ethanol and later scored under a benchtop microscope by an independent reader who was not aware of the study group. To measure pAkt's color intensity of immunoreactivity on the kidney sections, Image Scope software was applied.

The H-score was calculated based on the percentage of pAkt-positive cells according to the formula: H-score = 0 × (no stain) +1× (% of weak positive stain) + 2× (% of positive stain) + 3× (% of strong positive stain). The staining intensity was divided into four categories (0, 1, 2, 3), and the final score was determined by summing the results of multiplying the staining intensity by the proportion of stained cells as illustrated in the aforementioned equation.

2.11. Real-Time PCR Analysis

To assess the expression levels of the PI3K gene, we utilized a real-time PCR approach. Initially, total RNA was extracted from the renal tissues of the study's mice, utilizing an RNA extraction kit from Solarbio, China. For the real-time PCR, two different reactions were set up: one serving as the negative control (RT- mix) and the other as the sample reaction (RT+ mix). Specifically, the RT+ mix was composed of 10 µl of 2X RT buffer, 1 µl of 20X enzyme mix, and 9 µl of a mixture containing 1 µg of total RNA, topped up with nuclease-free water to reach a final volume of 20 µl. RT- mix replicated the composition of RT+ mix but with nuclease-free water replacing the enzyme mix. The extracted RNA concentration was then precisely determined with a Nanodrop spectrophotometer produced by Nanodrop Technologies, USA.

After determining the RNA concentration, complementary DNA (cDNA) was generated with an RNA-to-cDNA conversion kit purchased from Solarbio, China. The analysis of the gene expression was performed using a CFX-96 machine by Bio-Rad Laboratories, Inc., USA, supported by Promega, USA SYBR Green Master Mix to identify. PCR thermal cycle condition was: initial denaturation of 95°C for 30 seconds, 40 cycles with

denaturation of 95°C for 1 second, annealing at 60°C for 20 seconds, and elongation at 72°C for 20 seconds. For the calculation of expressional change, we employed the comparative CT method, or ΔΔCT method. The initial step was to determine the ΔCT value of both the GOI and the internal control by subtracting the CT value of the EC from that of the GOI with the help of the following formula:

ΔCT = CT (GOI) – CT (EC).

This ΔCT value is the relative quantity of expression of the gene of interest normalized to the internal control. To assist the comparison of levels of gene expression between samples or conditions, we further went ahead and computed the ΔΔCT by subtracting the ΔCT of the control sample from the ΔCT of the experimental sample. The fold change in gene expression was then calculated using the following equation:

Fold change = 2^-ΔΔCT.

For PI3K gene analysis, specific primers were used for amplification and detection. GAPDH was used as endogenous control gene. Full primer sequences, lengths, melting temperatures (Tm), GC content, and product sizes are listed in Supplementary Table 1.

Supplementary Table 1. Primer sequences used for quantitative real-time PCR analysis of **PI3K** and the endogenous control **GAPDH**. Primer length, GC content, estimated melting temperature (Tm), and expected product size are shown.

Gene	Primer	Sequence (5′–3′)	Length (bp)	GC Content (%)	Tm (°C)*
PI3K	Forward	CTCTCCTGTGCTGGCTACTGT	21	57	~66
	Reverse	GCTCTCGGTTGATTCCAAACT	21	52	~64
GAPDH	Forward	GGAGTCAACGGATTTGGT	18	50	~54
	Reverse	GTGATGGGATTTCCATTGAT	20	40	~56

This methodology ensures precise measurement and comparison of PI3K gene expression in renal tissues, contributing valuable insights into the molecular mechanisms under investigation (Al-Awaida *et al.*, 2023).

2.12. Statistical Evaluation

Statistical analysis was conducted via GraphPad Prism8.0 software (GraphPad Software, La Jolla, CA). The data accrued from this study met the standards for normality between all the groups, allowing us to compare using parametric tests. Data were gathered from four distinct experiments, with five replicates in each experiment, and are represented as mean ± SD. Differences were assessed using a one-way analysis of variance (for multiple groups) followed by Tukey's multiple comparisons test and student's t-tests. The threshold for statistical significance was set at $p < 0.05$.

2.13. Ethics approval and consent to participate

The Animal Care and Use Committee at the University of Kufa, Iraq reviewed and approved all experimental procedures under license number 15791.

3. Results

3.1. Differential Effects of Experimental Conditions on Creatinine and Urea Levels in Control, Vehicle, Sham, and TQ Groups: A Comprehensive Comparative Analysis

Our study meticulously investigated the impacts of various experimental conditions on creatinine and urea levels across four distinct groups: Control, Vehicle, Sham, and Thymoquinone (TQ). We executed pairwise comparisons among these groups to assess the statistical significance of the observed differences, ensuring a clear and detailed distinction of findings from each test.

3.2. Creatinine Levels Comparative Analysis:

In the initial phase of our analysis, we focused on creatinine levels. The Control group presented a mean creatinine level of 1.586±0.09, which was not significantly different from the Vehicle group's level of 1.554±0.11 (p=0.91), as illustrated in Figure 1B. This initial comparison indicates a baseline similarity between Control and Vehicle groups regarding creatinine levels.

Contrastingly, a significant deviation was observed when the Control group was compared with the Sham and TQ groups. The Sham group showed a dramatically lower mean creatinine level of 0.336±0.03 ($p<0.0001$), and similarly, the TQ group displayed a reduced mean creatinine level of 0.76±0.035 ($p<0.0001$) in comparison to the Control group. These findings suggest a notable protective impact of TQ on creatinine levels. Additionally, when compared to the Sham and TQ groups, the Vehicle group exhibited significantly elevated creatinine levels ($p<0.0001$ for both comparisons), further emphasized in Figure 1B.

3.3. Urea Levels Comparative Analysis:

Parallel to our creatinine level analysis, we examined urea levels across the groups. The Control group's mean urea level was 82.2±1.92, closely matched by the Vehicle group's mean level of 82.8±3.11 (p=0.98), as depicted in Figure 1A. This consistency underscores a similar baseline between these two groups in terms of urea levels as well.

However, a divergent pattern emerged when comparing the Control and Vehicle groups to the Sham group, which exhibited significantly reduced mean urea levels of 31.8±2.39 ($p<0.0001$). The TQ group also demonstrated lower mean urea levels of 53.2±2.65 ($p<0.0001$) in comparison to the Control and Vehicle groups, as highlighted in Figure 1A. These outcomes indicate a significant influence of the conditions represented by the TQ group on urea levels, pointing to the potential protective or modulatory role of TQ.

The results of our comparative analysis clearly indicate the differential impacts of experimental conditions on creatinine and urea levels across the Control, Vehicle, Sham, and TQ groups.

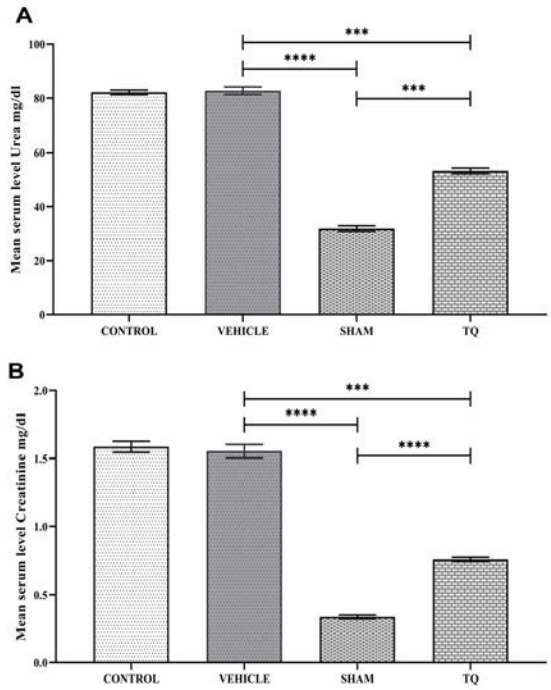

Figure 1: Comparison of Creatinine and Urea Levels among Experimental Groups. Panel A depicts mean ± SD Creatinine levels among Control, normal saline (vehicle), Sham, and TQ groups, showing no significant difference between Control and DMSO (p=0.9054) but they markedly elevated in comparison with sham group. TQ group revealed a substantial decrease as compared with Control and vehicle groups (****). Panel B presents urea levels for the same groups, mirroring the pattern in Creatinine, with Control and vehicle groups showing no difference (p=0.9805) but they considerably increased as compared with Sham group (****). Significant reduction is observed in TQ group as compared with Control and vehicle groups (****). Significance denoted as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.4. Differential Sham, CLP, and Thymoquinone (TQ) Treatment Effects on IL-6 and TNF-α Levels and TNF-α Receptor Expression

Our experiment extensively explores the effects of Sham, Cecal Ligation and Puncture (CLP), and Thymoquinone (TQ) treatments on the regulation of IL-6 and TNF-α levels, as well as TNF-α receptor expression. This section summarizes the findings across these biomarkers, highlighting the differential effects of each treatment regimen.

3.5. IL-6 Concentration Alterations:

The experiment began with the determination of the level of Interleukin-6 (IL-6) in experimental groups. The vehicle group and control group recorded very comparable IL-6 levels (1300±158.11 pg/mL vs. 1290±97.97 pg/mL, respectively) without any disparity noticed (P=0.99), which proves the absence of effect of normal saline, a standard solvent utilized in biological studies, on IL-6 levels. The result is presented in Figure 2A.

Contrastingly, significant reductions in IL-6 concentrations were noted in both the Sham (200±15.81 pg/mL) and TQ (306±15.57 pg/mL) groups compared to the control group, with all comparisons reaching statistical significance (P<0.0001). These groups also significantly lowered IL-6 levels compared to the vehicle group (P<0.0001). However, no notable variance was detected between the Sham and TQ groups in their effectiveness at reducing IL-6 levels (Sham vs. TQ: P=0.3189), indicating a similar impact on IL-6 modulation.

3.6. TNF-α Level Variations:

Subsequently, we examined the alterations in TNF-α levels under the influence of different treatments. As illustrated in Figure 2B, the control and vehicle groups exhibited comparable TNF-α levels (449.8±14.66 pg/ml and 444.2±17.42 pg/ml, respectively; P=0.99), indicating no significant effect of vehicle treatment on TNF-α levels. Interestingly, the TQ group manifested a significant decrement in TNF-α levels in comparison to both control and vehicle groups (P<0.0001), highlighting Thymoquinone's potential in mitigating inflammatory responses post-CLP.

3.7. TNF-α Receptor Expression Dynamics:

Finally, the assessment of TNF-α receptor concentrations revealed no significant difference between the control (800±50.12 pg/mL) and vehicle (790±38.89 pg/mL) groups (P=0.9864), as shown in Figure 2C. This outcome suggests the neutrality of vehicle treatment on TNF-α receptor levels. In stark contrast, the TQ group exhibited a significant reduction in TNF-α receptor levels in comparison to both the control and vehicle groups (P<0.0001). Yet, similar to the pattern observed with IL-6 levels, no significant disparity was noted between the Sham and TQ groups regarding their impact on TNF-α receptor levels (Sham vs. TQ: P=0.78).

Collectively, these findings delineate the distinct and significant impacts of Thymoquinone treatment on both IL-6 and TNF-α levels, as well as TNF-α receptor expression, in comparison to untreated and vehicle-treated controls.

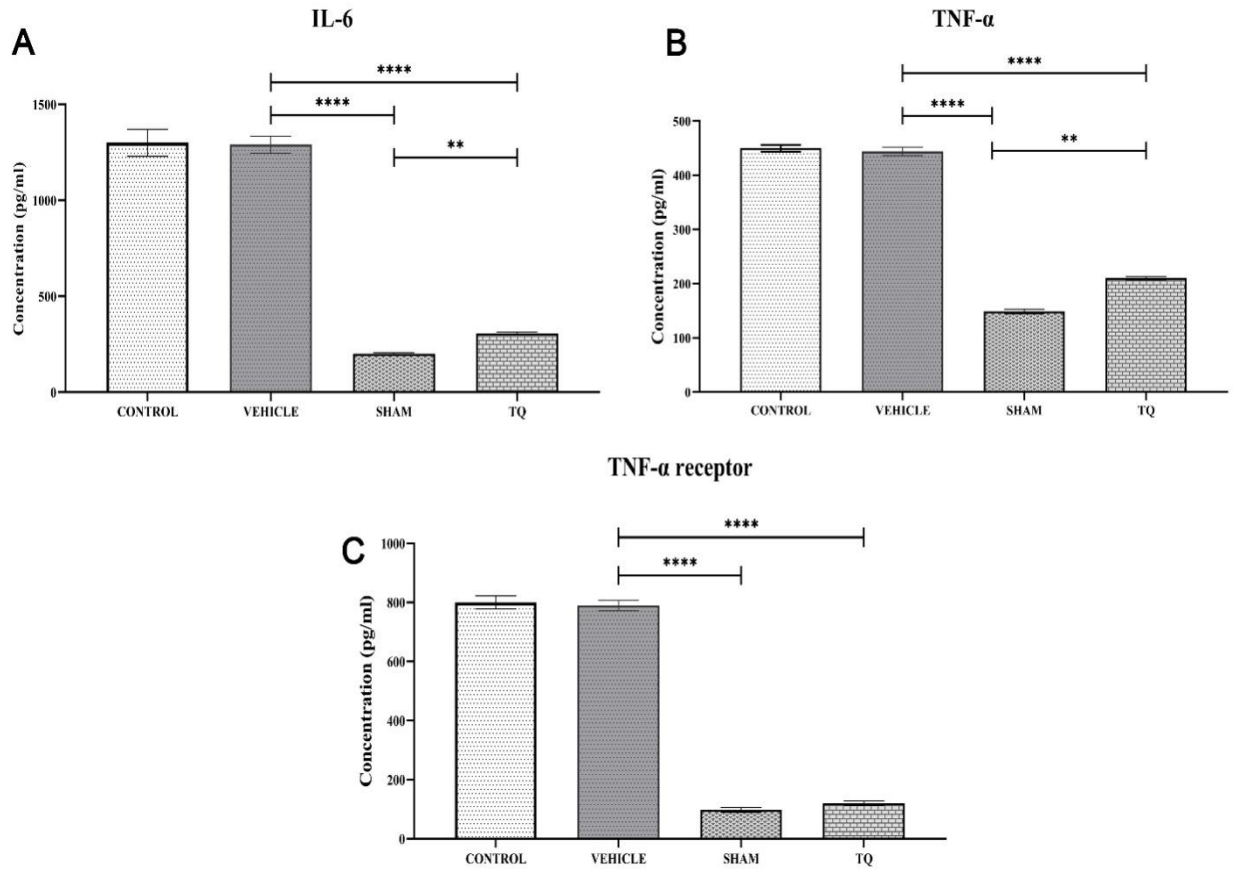

Figure 2: Effects of Various Treatments on IL-6 and TNF-α Levels and TNF-α Receptor Expression. Panel A illustrates IL-6 concentrations across Control, vehicle, Sham, and TQ groups. No significant difference exists between Control and vehicle (P=0.9997) but both showed significant elevations as compared with Sham group. TQ group shows a substantial reduction in IL-6 levels contrasted to both control and vehicle groups (P<0.0001). Panel B outlines the TNF-α level for the same groups. Control and vehicle groups display similar levels (P=0.9998) but they notably increased as compared with Sham group (****), whereas the TQ group exhibits significant reductions in comparison with Control and vehicle (P<0.0001). Panel C indicates TNF-α receptor concentrations, with control and vehicle showing no difference (P=0.9864), while they markedly increased as compared with Sham group. TQ group displays a significant reduction as compared with Control and vehicle groups (P<0.0001). Data are expressed as mean ± SD. Significance is depicted as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.8. Distinct Influence of Sham, CLP, and Thymoquinone (TQ) on IL-10 and F8-Isoprostane Levels: Indications for Oxidative Stress Reduction and Immunomodulation

Our study meticulously evaluated the differential impacts of sham operation, Cecal Ligation and Puncture (CLP), and Thymoquinone (TQ) treatment on IL-10 and F8-Isoprostane levels, which are pivotal markers for immunomodulation and oxidative stress, respectively. The following paragraphs detail our findings, correlating each to the appropriate figures.

3.9. IL-10 Levels Analysis:

In the comparative analysis of IL-10 levels, the control and vehicle groups showed no statistically significant difference (230±15.81 pg/mL vs. 234.4±23.80 pg/mL, P=0.99), suggesting that the solvent employed in these experiments (normal saline) does not significantly affect IL-10 levels. This observation is visualized in Figure 3A.

Contrastingly, the TQ treatment group demonstrated a marked increase in IL-10 levels, reaching 452±37.68 pg/mL, which represents a statistically significant elevation compared to both the control and vehicle groups (P<0.0001). This significant increase underscores the potent immunomodulatory effect of Thymoquinone, suggesting its role in enhancing anti-inflammatory responses. The comparative increase in IL-10 levels between the TQ and vehicle groups further solidifies the specific augmenting effect of Thymoquinone on this cytokine.

3.10. F8-Isoprostane Levels Analysis:

The examination of F8-Isoprostane levels, a marker for oxidative stress, revealed no significant variance between the control (87±3.80 pg/mL) and vehicle groups (86.6±6.107 pg/mL, P=0.99), indicating that the vehicle substance does not significantly influence oxidative stress markers in this context. This data is presented in Figure 3B.

However, a stark contrast was observed in the TQ treatment group, which exhibited a significant reduction in F8-Isoprostane levels (41±3.93 pg/mL, P<0.0001) compared to the control group. This pronounced decrease indicates that TQ treatment significantly mitigates oxidative stress, as measured by F8-Isoprostane levels. Furthermore, when compared to the vehicle group, the TQ group also displayed a significant reduction in F8-Isoprostane levels (P<0.0001), highlighting the substantial oxidative stress reduction afforded by Thymoquinone treatment.

Our findings distinctly indicate that Thymoquinone treatment significantly amplifies IL-10 levels, thereby potentially enhancing anti-inflammatory responses. Concurrently, TQ treatment considerably lowers F8-Isoprostane levels, indicating a reduction in oxidative stress.

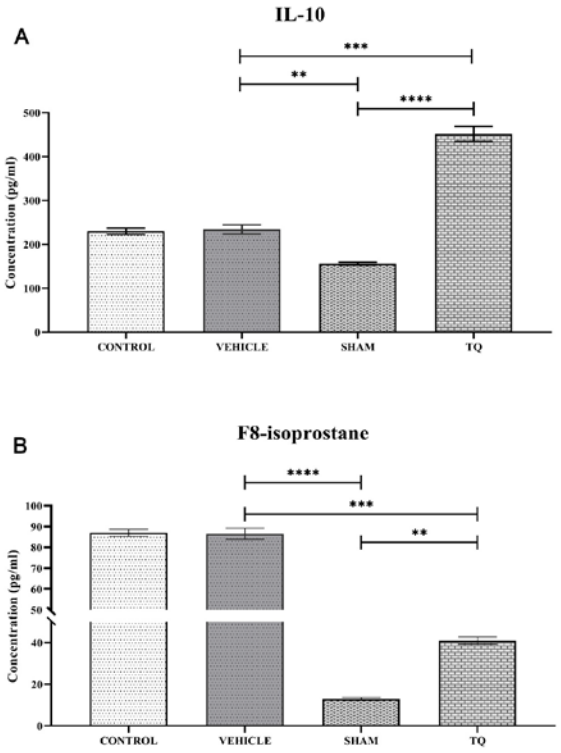

Figure 3: Evaluation of IL-10 and F8-Isoprostane Levels across Experimental Groups. Panel A shows IL-10 levels in control, vehicle, Sham, and TQ groups. No significant difference between control and vehicle groups (P=0.9976), but significant increase in TQ group (P<0.0001) is observed as compared with Control, vehicle and Sham groups. Panel B depicts F8-isoprostane levels among the same groups, showing no difference between Control and vehicle groups (P=0.9999), but they show substantial increases as compared with Sham group. TQ treatment showed a significant reduction in F8-isoprostane levels compared to Control and vehicle groups (P<0.001 for both). Data are represented as mean ± SD. Significance indicated as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.11. Influence of Thymoquinone on pAkt Antibody Expression in Renal Tissue Post-CLP

This section of the study extensively inspected the impact of Thymoquinone (TQ) on the renal tissue expression of pAkt antibody following Cecal Ligation and Puncture (CLP) via immunohistochemistry processes for critical analysis.

3.12. Immunohistochemistry Observations:

Our initial assessment documented a considerable disparity in pAkt antibody immunoreactivity across diversified experimental groups. Precisely, the vehicle-treated and CLP control groups both presented weak pAkt immunoreactivity, as clearly demonstrated by less intensively brown-pigmented renal tissue sections (Figures 4A, 4B, and 4C). Quantitatively, this was accompanied by the mean H-score, indicating a statistically significant reduction in pAkt expression in these groups compared to the Sham group, affirming the toxic impact of CLP ($P \leq 0.05$, Figure 4E).

3.13. Effect of Thymoquinone:

When compared to control and vehicle groups, Thymoquinone (TQ) pretreatment showed an apparent increase in the pAkt antibody immunoreactivity in the kidney tissues. Such an increase was visually evident as denser brown staining in TQ-treated group tissue sections (Figure 4D). To complement this qualitative observation, the H-score analysis also statistically quantified the increase with greater mean levels of pAkt expression in the TQ-treated group compared to the control CLP group ($P \leq 0.05$, Figure 4E).

These findings propose an important role played by Thymoquinone in increasing the immunoreactivity of pAkt antibody in renal tissues following CLP. This increase indicates a potential protective mechanism employed by TQ to counteract renal alterations induced by CLP. In view of the significance of these observations, further research is an imperative necessity to dissect the precise molecular mechanisms whereby TQ provides protection and to assess the potential clinical relevance of these observations.

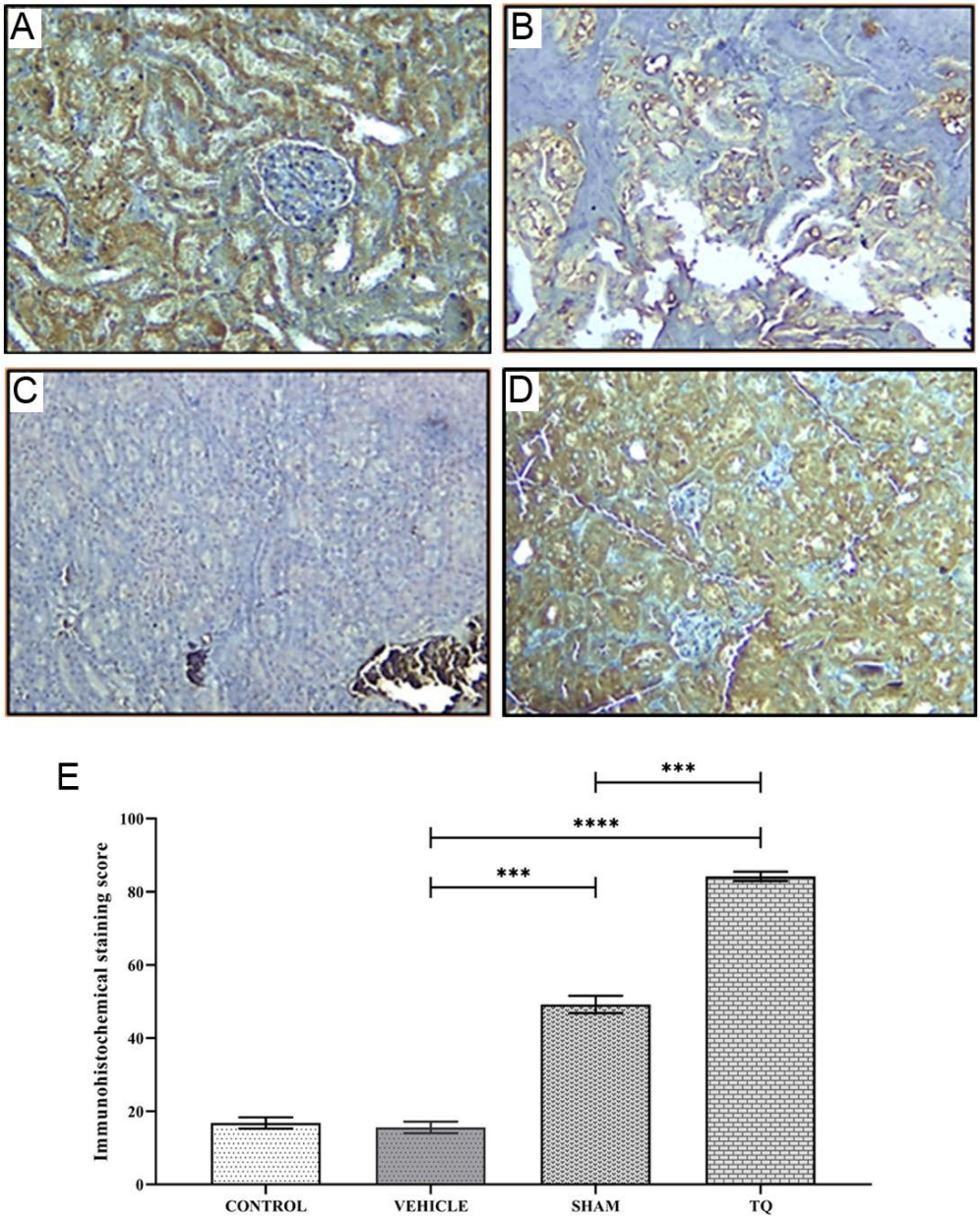

Figure 3: Thymoquinone Augments pAkt Antibody Expression in Renal Tissue Post-CLP. Immunohistochemical staining for pAkt was conducted in different groups: Sham (Figure 4A), Control (Figure 4B), vehicle (Figure 4C), and Thymoquinone (TQ)-treated (Figure 4D). Brown staining indicates pAkt antibody expression. The mean H-scores are presented in Figure 4E. Data are expressed as mean ± SD. Significance is depicted as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.14. Thymoquinone: Comparative Effects on PI3K Gene Expression Following Post-CLP

We closely looked at the impact of Thymoquinone (TQ) on PI3K gene expression following the CLP operation, as depicted in Figure 5. This study intended to clarify the potential modulatory action of TQ on PI3K gene expression, which is a crucial molecule in cell signaling pathways included in cell growth, proliferation, differentiation, motility, survival, and intracellular transport.

3.15. Control vs. Vehicle Group Comparison:

Our primary comparison between the control group, which received CLP alone but no other treatment (6.14±0.27), and the vehicle group, which received an inert treatment that was assumed to be inactive (6.04±0.18; P=0.89), did not reveal any significant difference in PI3K gene expression levels. This suggests that the vehicle in our experimental setup does not show any notable effect on PI3K gene expression levels, so it is an effective baseline to compare with other groups.

3.16. Effect of Thymoquinone on PI3K Gene Expression:

Remarkably, when we compared the PI3K gene expression levels of the control group with those of the Sham group (2.12±0.24) and the TQ group (2.156±0.19), we observed a highly significant decrease in PI3K gene expression in the TQ group, with P-values of less than

0.0001. This pronounced downregulation of PI3K gene expression in the TQ group, as opposed to the control group's levels, underscores the potent modulatory effect of TQ treatment on this critical gene's expression following the CLP operation.

3.17. Sham vs. TQ Group Comparison:

Further analysis between the Sham group and the TQ group revealed no significant difference in PI3K gene expression levels (P=0.99), indicating that TQ administration results in PI3K gene expression comparable to that observed in the Sham group. This parity between the Sham and TQ groups suggests that TQ treatment effectively mimics the basal level of PI3K gene expression observed in the absence of the CLP-induced stress condition.

Our findings evidently indicate that Thymoquinone treatment significantly enhances PI3K gene expression following the Cecal Ligation and Puncture operation. The results show a strong example for the role of TQ in modifying PI3K gene expression, one of the mechanisms via which it might be applying the therapeutic effects in stress or post-surgery situations. Action mechanisms of these effects can be inspected further to fully clarify the effects of TQ on cellular signaling pathways and its therapeutic applications.

3.18. Thymoquinone Mitigative effect on Kidney Tissue Morphology Following the Cecal Ligation and Puncture Procedure

This part of our study ackles evaluating the therapeutic capacity of Thymoquinone (TQ) in maintaining kidney tissue integrity after Cecal Ligation and Puncture (CLP) surgery, an experimental procedure for inducing sepsis in research. Histopathological assessment of kidney tissues from various groups provided an indication of TQ's protective effect against CLP-induced renal damage.

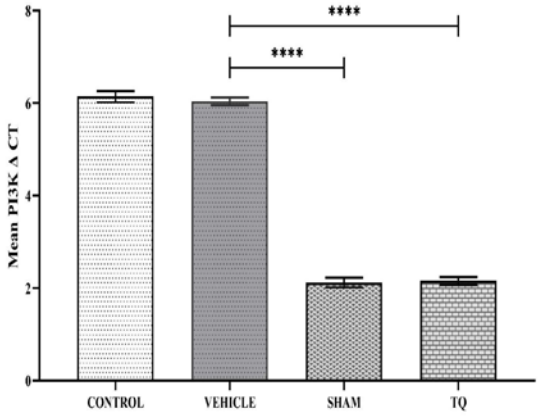

Figure 4: Effects of Various Treatments on PI3K Gene Expression. This figure displays the comparative analysis of PI3K gene expression in different groups: Control, vehicle, Sham, and Thymoquinone (TQ)-treated. The gene expression levels are denoted as arbitrary units on the y-axis. Data are expressed as mean ± SD. Significance is depicted as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.19. Sham Group Morphology:

The morphology of kidney tissues in the Sham group initially revealed normal renal epithelium morphology, illustrating an absence of significant morphological changes due to the sham procedure itself, as seen in Figure 6A. This set a baseline for determining the degree of tissue damage and TQ efficacy in the experimental model..

3.20. CLP and Vehicle-Treated Group Results:

Tissues collected from mice that were subjected to the CLP surgery, regardless of whether they were treated with a vehicle, displayed severe pathological features. These characteristics included vascular congestion, the presence of necrotic lesions, eosinophilic cytoplasm, and densely packed nuclei, as depicted in Figures 6B and 6C. Such findings underscore the severe renal damage inflicted by the CLP procedure, establishing a context for assessing TQ's protective potential.

3.21. TQ-Treated Group Observations:

Remarkably, kidney sections from the TQ-treated group exhibited a distinctively milder degree of tissue alteration. These sections showed moderate hypertrophy of renal tubules along with mild lesions, as illustrated in Figure 6D. This observation suggests a pronounced protective effect of TQ, mitigating the harsh impact of the CLP operation on renal tissues. The comparatively less severe morphological changes in the TQ-treated group highlight the potential of TQ as a protective agent against CLP-induced kidney damage.

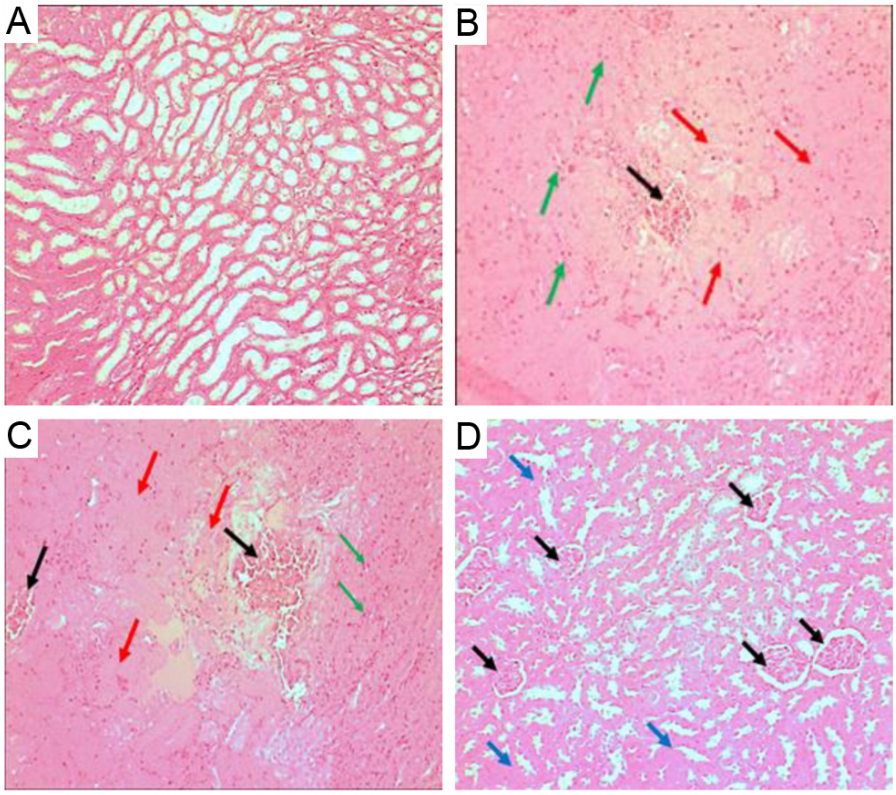

Figure 5: Evaluation of Thymoquinone's Mitigative Effects on Kidney Tissue Morphology Post-CLP. This provides histopathological images representing the differential impact of Thymoquinone (TQ) on kidney tissue morphology following the cecal ligation and puncture (CLP) procedure. Tissue sections are stained for structural analysis and examined under a microscope. The scale bar represents a standard length for comparative analysis. In Figure 6A, the Sham group exhibits typical renal epithelium, denoting no significant structural alteration as a consequence of the sham procedure. However, kidney tissues from CLP-subjected mice, regardless of vehicle treatment, display considerable pathological changes. These alterations, which include vascular congestion, necrotic lesions, eosinophilic cytoplasm, and dense nuclei, are evident in Figures 6B and 6C, suggesting severe renal damage inflicted by the CLP procedure. In a contrasting manner, renal tissue sections from the TQ-treated group, as shown in Figure 6D, depict moderate hypertrophy of renal tubules and mild lesions. These relatively mild morphological changes suggest a potential mitigative effect of TQ on CLP-induced renal damage.

Finally, our data map out a specific protective response of Thymoquinone in preserving morphology of kidney tissues following the CLP surgery. The seemingly quantitative contrast in damage severity between CLP and TQ-treated group supports the potential therapy of TQ. Even so, still justified is closer study of precise mechanisms by which TQ operates with these protecting activities. Future research studies should aim to clarify these mechanisms, hopefully culminating in therapeutic applications of TQ in mitigating renal injury in sepsis or other similar conditions.

4. Discussion:

Although several studies have accounted for the antioxidative and anti-inflammatory activities of Thymoquinone, our study sheds more light by examining its impact on the PI3K/Akt pathway in acute kidney injury induced by sepsis. Through a holistic integration of histological, biochemical, and molecular data, this study contributes to the burgeoning literature on the potential mechanisms of action of TQ in renoprotection.

Sepsis, a clinical condition involving the host's amplified inflammatory response to infection, is well recognized to have severe harmful effects on renal function, resulting in acute kidney injury. Despite the great progress in the development of novel therapies to arrest sepsis-induced morbidity and mortality, it remains a universal problem that requests ongoing research (van der Poll *et al.*, 2021; Shalan *et al.*, 2024). Our results, showing elevated serum levels of urea and creatinine in septic mice after-cecal Ligation and Puncture (CLP), confirm the kidney damage frequently seen in sepsis and support the usefulness of the CLP model for sepsis induced AKI research (Kim *et al.*, 2022). Furthermore, existing research highlights TQ's effectiveness in reducing such biomarkers, as revealed by renal protective effects in glycerol induced acute kidney injury in rats. AKI is defined by the rapid decrease in glomerular filtration rate and the accumulation of nitrogenous waste, leading to severe kidney damage (Bilgili *et al.*, 2014).

Increase in serum levels of IL-6, TNF-α, and TNF-α receptors in the CLP group, in contrast to the sham group, displays the role of an augmented inflammatory response in sepsis. This augmentation aligns with previous reports representing increases in these cytokines at sepsis models (Shou *et al.*, 2023). The efficiency of Thymoquinone (TQ) in markedly reducing these inflammatory mediators reinforces its therapeutic capability, reasonable with findings from studies showing TQ's capability to modulate similar markers in different inflammatory diseases (Zanders *et al.*, 2022).

The change of TNF-α via TQ, critical due to its role in improving IL-6 production and facilitating inflammatory processes, apoptosis, and oxidative damage, supports reports demonstrating TQ's inhibition of the NF-κB pathway and consequent reduction of TNF-α and IL-6 production (Venkataraman *et al.*, 2021). Though, the extent of cytokine decrease differs across studies, probably due to changes in TQ dosing, administration timing, or the sepsis models retained. These differences highlight the complexity of septic reaction and the need for standardization in evaluating therapeutic interventions.

In contrast to our results on TNF-α receptor modulation by TQ, some studies report no considerable changes in receptor expression in different disease models treated with TQ (Alasmari *et al.*, 2023), suggesting that TQ's effect on the TNF-α receptor might vary with the original pathology. This designates a nuanced mechanism of action for TQ that necessitates further investigation.

The detected increase in IL-10 serum levels in the CLP group, which was amplified by TQ pretreatment, supports results from preceding studies demonstrating the anti-inflammatory effects of IL-10 in sepsis models (Guo *et al.*, 2020). IL-10 is noted to play a very significant part in governing the inflammatory response, suggesting a protective mechanism that has the possibility to improve outcomes in septic states (Vivas *et al.*, 2021). This surge in IL-10 following TQ administration implies that TQ may improve the body's natural antiinflammatory response to sepsis.

Though, the elevated IL-10 levels in TQ-treated mice, exceeding those observed in septic mice without TQ treatment, give an interesting contrast to findings by researchers in 2022, who reported lower IL-10 levels in septic mice following TQ treatment in comparison to untreated septic mice (Alkharfy *et al.*, 2018). This difference could be accredited to differences in experimental design, such as the timing of TQ administration in relation to the onset of sepsis, the dosage of TQ used, or the specific strains of mice used in the studies. Such variables could considerably impact the immunomodulatory effects of TQ, showcasing the need for further research to summarize the ideal conditions under which TQ exerts its maximal therapeutic benefit.

Additionally, the mechanism by which TQ raises IL-10 levels highlights the need for further investigation. Given that TQ has been revealed to modulate numerous signaling pathways related to inflammation and immune response regulation (Darakhshan *et al.*, 2015), it is probable that TQ's action on these pathways may indirectly lead to amplified IL-10 production. This hypothesis aligns with the wider literature on the immunomodulatory outcomes of natural compounds, which often include complicated interactions with the host's immune system (Alkharfy *et al.*, 2018).

Granted these results, our research contributes to the growing amount of data indicating that TQ has potential as a treatment for sepsis, especially because of to its ability to influence the inflammatory response through cytokines such as IL-10. The observed variances with prior studies, nevertheless, emphasize the intricacy of the immune response in sepsis and the influence of several experimental variables on the results of such studies. To entirely understand the circumstances under which TQ can be used to treat sepsis and other inflammatory illnesses, more research is needed.

Oxidative stress and the production of free radicals play vital roles in the pathogenesis of sepsis and the resultant inflammatory response. In our study, the higher levels of F8-isoprostane in the renal tissues of septic mice, in comparison to the sham group, emphasize the impact of oxidative stress in sepsis induced renal injury. The substantial reduction in these levels following TQ treatment proposes its possible antioxidative properties, associating with preceding studies that have highlighted TQ's ability to mitigate oxidative stress markers in various models of disease(Ow *et al.*, 2021). This is steady with findings from Linillos-Pradillo et al., who stated a decrease in oxidative stress markers, comprising F8-isoprostane, in a model of ischemia-reperfusion injury treated with TQ (Linillos-Pradillo *et al.*, 2023).

Addionally, antioxidative activity of TQ, particularly its effect on F8-isoprostane, clarifies its protective mechanisms against renal injury in sepsis. This is reinforced by research from Tiba et al., demonstrating TQ's broad antioxidative outcomes in a diabetic nephropathy model, viewing a decrease in renal oxidative stress markers along with enhancements in renal function (Tiba *et al.*, 2023).

Our findings support other studies on renoprotective activity of TQ against various models of renal damage, including glycerol-induced and cisplatin-induced nephrotoxicity (Sayed and Morcos, 2007; Bilgili *et al.*, 2014). The same reductions in IL-6 and TNF-α upon TQ administration have been demonstrated in models of LPS-induced inflammation (Ojha *et al.*, 2015). However, few studies have touched upon its modulation of the PI3K/Akt signaling pathway during polymicrobial sepsis, highlighting the unique emphasis of our work. Contrary to findings of Qu et al.,(Qu *et al.*, 2020) which saw suppression of PI3K/Akt in sepsis in the kidneys, our results confirm that TQ has the capability to revive such a signaling pathway and may be a viable targeted therapeutic intervention.

Although TQ's antioxidative properties are extensively recognized, variations in the extent of its consequences amongst findings may result from diverse investigational models, dosage differences, or timing of administration. These alterations underline the many-sided antioxidative mechanisms of TQ and the complicated nature of oxidative stress in sepsis.

Furthermore, TQ represents a promising therapeutic option in reducing sepsis induced renal injury through antioxidative activity. Additional studies need to be conducted to fully reveal the exact mechanisms by which TQ performs to protect and to discover its therapeutic value in a wider variation of septic diseases.

The PI3K/Akt signaling pathway has been recognized as a significant mechanism in sepsis pathogenesis and a therapeutic target. In our study, a marked decrease in the mRNA expression of PI3K and phosphorylated Akt in septic mice was detected when compared to controls, revealing the pathway suppression during sepsis. Of special relevance, TQ administration obviously reduced these parameters, showing its modulatory effect on the PI3K/Akt signaling pathway, that may have a role in improving septic renal damage. The current finding is reinforced by the outcome of Qu et al., showing reduced

levels of phospho-Akt and phospho-PI3K in septic rats' kidneys, underlining the dysfunction of the pathway in sepsis (Qu *et al.*, 2020).

Activation of the PI3K-Akt pathway has been shown to have defensive effects against endotoxemia via the suppression of proinflammatory cytokines, decreasing coagulation, and improving survival in sepsis models (Qu *et al.*, 2020). This defensive act is also shown in an acute spinal cord injury model, where TQ treatment improved PI3K and phospho-Akt levels, denoting its potential as a protective agent against renal injury (Junaid *et al.*, 2021). The similarity of our results with such studies complements the therapeutic value of inhibiting the PI3K/Akt pathway in sepsis.

However, various investigations may have distinct results on the protective effect of TQ in sepsis induced kidney injury and its degree of impact on the PI3K/Akt pathway. This could be due to the differences in the experimental model, TQ dosage, or the timing of the intervention. This variety demonstrates the complex participation of the PI3K/Akt pathway in the pathophysiology of sepsis and the intrinsic complexity of the disease.

The evidence proposes that modulating TQ through the PI3K/Akt signaling pathway is a promising new approach to diminish kidney damage carried on by sepsis. Nevertheless, further studies will help elucidate its detailed mechanisms of action.

5. Conclusion

This study provides robust experimental proof that Thymoquinone (TQ), the bioactive compound of *Nigella sativa*, possesses a potent therapeutic effect against sepsis-induced Acute Kidney Injury (AKI) by multi-level biology modulation. Our findings reveal that TQ pretreatment significantly improved renal function, as shown by the reduction in the levels of serum creatinine and urea, which were drastically elevated in septic CLP-induced mice. These functional improvements were supported by histopathological findings of remarkable renal architecture preservation and reduced tissue injury in TQ-treated mice compared to the CLP and vehicle groups.

Molecularly, TQ significantly down-regulated systemic inflammation via inhibition of pro-inflammatory cytokines IL-6 and TNF-α, and repression of TNF-α receptor expression. Concurrently, TQ up-regulated the anti-inflammatory cytokine IL-10, reflecting a desirable shift to an anti-inflammatory profile. Furthermore, TQ greatly lessened oxidative stress, as indicated by decreased F8-isoprostane levels in renal tissue. These findings render TQ a bifunctional agent that is capable of influencing inflammatory and oxidative processes, which are the centers of gravity in AKI pathology during sepsis.

Interestingly, our analysis of the PI3K/Akt signaling pathway, a crucial axis that regulates cell survival, apoptosis, and inflammation, revealed that TQ increases pAkt protein levels and normalizes PI3K gene expression back to sham levels. This regulation proves that TQ has at least partial renoprotective functions by restoring cellular signal balance lost during sepsis. This is novel mechanistic insight, positioning PI3K/Akt as a potential therapeutic target and TQ as a potential modulator of this pathway in septic AKI models.

These findings not only serve the study's objective to evaluate the therapeutic efficacy of TQ in septic AKI but also render TQ an excellent choice for future drug development. The breadth of its action—ranging from functional to molecular and histological protection—underscores its promise for translation to the clinic where sepsis-induced acute kidney injury remains a major cause of morbidity and mortality.

While these results are encouraging, additional preclinical studies are required to assess TQ's long-term safety profile, optimal dosing regimens, and pharmacokinetics in septic models. More investigation is also required to identify whether PI3K/Akt modulation by TQ has downstream effects on apoptosis, autophagy, and mitochondrial function. Furthermore, testing of TQ in combination with existing therapeutic agents may reveal synergistic effects that enhance outcomes in sepsis. Lastly, adequate clinical trials in humans shall be the determining factor towards proving the efficacy and safety of TQ as a drug against sepsis-associated AKI and, by default, other inflammatory organ injury.

6. Authors' Contributions:

Conceptualization, H.J. and W.A.; Data curation, K.G., H.A.-A., and I.H.; Formal analysis, H.J., Y.G., A.S., O.A.B., and N.H.; Investigation, H.J., B.M., H.Q., W.A., and S.S.Z.; Methodology, B.M., G.F., H.Q., W.A., B.S., Y.G., V.T., A.S., O.A.B., G.C., N.G., and N.H.; Resources, Y.G., V.T., A.S., A.A.A, A.A.H., T.M.R., T.S.A., and G.C.; Software, B.M., K.G., H.A.-A., and V.T.; Validation, H.J., B.M., G.F., K.G., and Y.G.; Visualization, B.M.; Writing – original draft, H.Q., H.A.-A., B.S., O.A.B., N.G., A.A.B., A.M.A., and I.H.; Writing – review & editing, H.J., W.A., S.S.Z., B.S., G.C., A.A.B., I.H., A.M.A., and N.H.

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