Journal of Chemical Health Risks

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JCHR (2025) 15(2), 1242-1252 | ISSN:2251-6727

Effect of Thymoquinone in the Regulation of Pro Inflammatory Cytokines and Pro Fibrotic Components during Sepsis Induced Acute Kidney Injury in Vivo

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(Received: 16 January 2025 Revised: 20 February 2025 Accepted: 31 March 2025)

KEYWORDS

Acute kidney injury;

Thymoquinone;

Inflammation;

Sepsis

ABSTRACT:

Introduction: Sepsis-induced acute kidney injury (AKI) is a severe condition driven by oxidative stress, inflammation, and fibrosis, often modelled using Lipopolysaccharide (LPS). Thymoquinone (TQ), a bioactive compound from Nigella sativa, is recognized for its antioxidant and anti-inflammatory properties, offering potential therapeutic benefits in mitigating LPS-induced AKI.

Objectives: This study investigates the protective effects of TQ (18 mg/kg), a cytoprotective active compound derived from Nigella sativa, against LPS-induced AKI. Specifically, the study aims to evaluate TQ's ability to reduce cellular oxidative stress and modulate the expression of profibrotic components (PAI-1, TGF-β, uPA, uPAR) and proinflammatory cytokines (IL-17A, IL-23, TNF-α).

Methods: The study involves biochemical estimation of BUN,creatinine,Gene expression analysis and Immunofluorescence assays.

Results: Both pre-treatment and post-treatment with TQ significantly ROS production. TQ also effectively regulated the expression of proinflammatory and profibrotic markers, mitigating oxidative stress and inflammation in LPS-induced AKI.

Conclusions: This study demonstrates that TQ exhibits potent protective effects against LPS-induced sepsis and AKI by reducing oxidative stress and modulating inflammatory and fibrotic responses.

1. Introduction

Acute Kidney (AKI) is a prevalent condition worldwide, affecting individuals regardless of economic status. It is linked to significant morbidity, mortality, and increased healthcare costs 1. AKI is currently the third leading cause of death among trauma patients, following bleeding and brain injuries 2. According to recent statistics, approximately 13.3 million people globally are affected by AKI each year, with 85% of these cases occurring in developing countries. Furthermore, it is

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JCHR (2025) 15(2), 1242-1252 | ISSN:2251-6727

estimated that AKI contributes to 1.7 million deaths annually. Up to 50% of critically ill patients develop AKI, which is associated with high rates of morbidity and mortality 1,1,3,4. Beyond short-term complications, such as fluid overload, electrolyte or acid-base imbalances, immune dysfunction, and bleeding, AKI also has long-term adverse effects on survival.

Renal inflammation leads to decreased renal blood flow and a reduced glomerular filtration rate (GFR). Chronic inflammation progressively diminishes GFR, eventually contributing to end-stage renal disease (ESRD). Both acute and chronic renal failure are characterized by elevated fibrinogen levels and reduced fibrinolysis as a result of renal damage 5–8. Current treatments for AKI include Renal Guard Therapy, dialysis, α-melanocyte–stimulating hormone, alkaline phosphatase, siRNA, THR-184, sodium-glucose cotransporter 2 (SGLT2) inhibitors, removal of catalytic iron, and medications like Sevelamer, Cinacalcet, and Jardiance to mitigate sustained declines in GFR. While no pharmacotherapy specifically targets AKI itself, some treatments address its complications. Medications play a crucial role in managing AKI, as changes in renal function and dialysis can lead to issues such as toxicities and underdosing. Hemodialysis and peritoneal dialysis are associated

with side effects, including low blood pressure, sepsis, muscle cramps, itchy skin, hernia, weight gain, insomnia, bone and joint pain, loss of libido, erectile dysfunction, dry mouth, anxiety, abdominal pain, fever, and chills. In kidney transplants, graft rejection remains a significant concern 9–11 . Consequently, there is growing interest in developing novel therapeutic agents for effective AKI management.

Several phytochemicals are known to offer protective effects for renal health. For instance, allicin has anti-inflammatory properties, baicalin reduces blood urea nitrogen (BUN) and creatinine levels, curcumin lowers TGF-β, IL-8, and creatinine levels, and epigallocatechin-3-gallate has antiproteinuric and anti-apoptotic effects 12,13,13–15. Thymoquinone (TQ), a major active component of Nigella sativa (black seed or black cumin), has demonstrated antioxidant, analgesic, antipyretic, antifungal, antibacterial, anticancer, anticonvulsant, hepatoprotective, and neuroprotective activities 16–18,18,18–23 . Notably, TQ has shown protective effects against renal damage by exerting anti-inflammatory, antioxidant, and anti-apoptotic activities. It has been found to protect against ischemia-reperfusion (IR)-induced renal damage by reducing profibrotic and proinflammatory cytokines 24 . This study aims to investigate the protective role of TQ in LPS induced AKI, focusing on the modulation of pro-inflammatory and profibrotic pathways.

2. Methods

ethical approval from the Institutional Animal Ethics Committee (IAEC) of Yenepoya University (Clearance No. YU/IAEC/20/2019). Healthy male C57BL/6 mice, aged 6–8 weeks and weighing 25 ± 5 g, were procured from Adita Biosystems, Karnataka, India (Registration No. U85100KA2015PTC079003). The mice were housed in polypropylene cages under a 12-hour light/dark cycle and provided with food and water ad libitum. The experimental set up includes four groups, control, LPS, LPS+ TQ pre-treatment & post treatment (n= 8) in each group. In the Control group, mice were intraperitoneal(i.p.) administration of saline (10 mL/kg, i.p.) on the first day. In the LPS Model group, a single LPS injection (10 mg/kg, i.p.) was administered on the first day. The Pre-Treatment group was received TQ (18 mg/kg, i.p.) for two days, followed by LPS (10 mg/kg, i.p.) on the third day, and was be sacrificed 72 hours post-LPS exposure. In the post-treatment group, mice were injected with LPS (10 mg/kg, i.p.) on the first day, followed by TQ (18 mg/kg, i.p.) on the second and third days, and sacrificed 72 hours post-LPS. Body weight was be recorded daily, and mice was be euthanized 24 hours after the final LPS injection using ketamine hydrochloride, with blood samples collected via cardiac puncture. Following the treatments, biochemical estimations were performed, and analyses of gene and protein expression were carried out.

Biochemical estimation of Creatinine and BUN

The estimation of Creatinine and BUN is critical for assessing kidney function and diagnosing renal diseases. Both are waste products filtered by the kidneys, and their levels in the blood reflect how well the kidneys are functioning.

Estimation of Creatinine

The creatinine estimation was done using the enzymatic Jaffe's method using kit (Product no :11009001), where creatinine reacts with picric acid to form a coloured

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compound, creatinine alkaline picrate. The change in absorbance is proportional to the creatinine concentration. The absorbance was read at 510 nm using a semi-auto analyser.

Estimation of Urea

Urea estimation was performed using the enzymatic Urease-Glutamate Dehydrogenase (GLDH) method with a commercial kit (Product No: 11412008). Urea is hydrolysed by urease to produce ammonia (NH_3) and carbon dioxide (CO_2). The resulting ammonia reacts with α-ketoglutarate in the presence of GLDH and NADH, which is oxidized to NAD^+. The reduction in NADH, directly proportional to urea concentration, is measured by the absorbance change at 340 nm.

Gene expression studies

The study evaluated the regulation of profibrotic components (PAI-1, TGF-β, uPA, uPAR, mTOR, α-SMA, Collagen -1,Fibronectin, MMP2 and MMP 9) and proinflammatory cytokines (IL-17A, IL-23, TNF-α) by analysing their mRNA expression in mouse kidneys using quantitative RT-PCR. RNA was isolated using TRIzol Reagent (Catalogue No. 15596018, Thermo Fisher), cDNA synthesis was performed with the PrimeScript™ RT Reagent Kit (Catalogue No. RR047A, Takara Bio), and qRT-PCR was conducted using SYBR® Premix Ex Taq™ II (Catalogue No. RR820A, Takara Bio). SYBR Green fluorescence was used for gene expression quantification, providing insights into the regulation of these genes in response to treatment.

Immunofluorescence - Protein Expression

For immunofluorescence protein expression analysis, tissue sections (4μm) were first fixed in 4% paraformaldehyde for 15-30 minutes and then washed with PBS. After deparaffinization in xylene and rehydration through graded ethanol solutions, antigen retrieval was performed using 10 mM citrate buffer (pH 6.0) at 95°C for 20 minutes, followed by cooling and PBS washing. The sections were blocked with 5% BSA in PBS for 1 hour to prevent non-specific binding. Primary antibodies for PAI-1, uPA, uPAR, and TGF-β were incubated overnight at 4°C, and after PBS washing, the sections were incubated with fluorescently labelled secondary antibodies for 1 hour. Nuclei were stained with DAPI for 5 minutes, and the sections were mounted with anti-fade mounting medium. Finally, protein expression was observed under a fluorescence microscope, and the intensity of fluorescence was quantified.

3. Results

In the present study we wanted to check the effect of the effect of TQ on AKI. The mice were exposed to LPS 10 mg/kg) and later treated with TQ, (18mg/kg,ip) through intraperitoneal injection. The C57BL/6 mice exposed to LPS showed increased expression of creatinine & BUN as compared to the control group, whereas mice exposed to LPS+ TQ showed decreased expression of creatinine & BUN indicating the protective action of TQ on LPS induced AKI (Figure-1). Further, mice treated with LPS, showed increased expression ofIL-17A and IL-23, whereas LPS +TQ treated mice showed decreased expression of IL-17A and IL-23. LPS treated mice also showed increased expression of TNF-ά and TGF-β, whereas LPS + TQ treated mice showed decreased expression of TNF-ά and TGF-β, indicating that TQ has controlling effect on pro-inflammatory cytokines during LPS induced AKI. (Figure-2).

Later we checked for the involvement of fibrinolytic system during in AKI. In this study the mice were exposed to LPS and the treated with TQ. We observed that LPS treated mice showed decreased expression of uPA and uPAR, Whereas LPS + TQ treated mice showed increased expression of uPA and uPAR, further LPS exposure showed increase expression of PAI-1 in those mice, however, treatment with TQ could able to decrease the expression levels of PAI-1, indicating that the TQ could reverse the fibrinolytic system during AKI (figure-3).

Further to dissect the pathways responsible in this process, we checked the expression levels of mTOR and α-SMA. We found that LPS treated mice showed increased decreased expression of mTOR and α-SMA, whereas treatment with TQ (LPS+TQ) showed decreased expression of mTOR and α-SMA, indicating the mTOR pathway may be involved in LPS induced AKI. We also wanted to seek if any sought any fibrotic expression during AKI. So we exposed mice with LPS and then treated with TQ and we found that mice exposed to LPS showed increased expression of Collagen-1 and Fibronectin, whereas LPS +TQ treatment showed decreased expression of Collagen-1 and Fibronectin, indicating the appearance of fibrotic components has

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JCHR (2025) 15(2), 1242-1252 | ISSN:2251-6727

started during AKI. However TQ treatment could able to reverse the fibrotic component during LPS induced AKI (Figure-4).Further immunofluorescence study supported the expression analysis observation showing the reversal of TGF-β and the fibrinolytic system (increased uPA and uPAR and decreased PAI-1) during AKI (Figure 5).

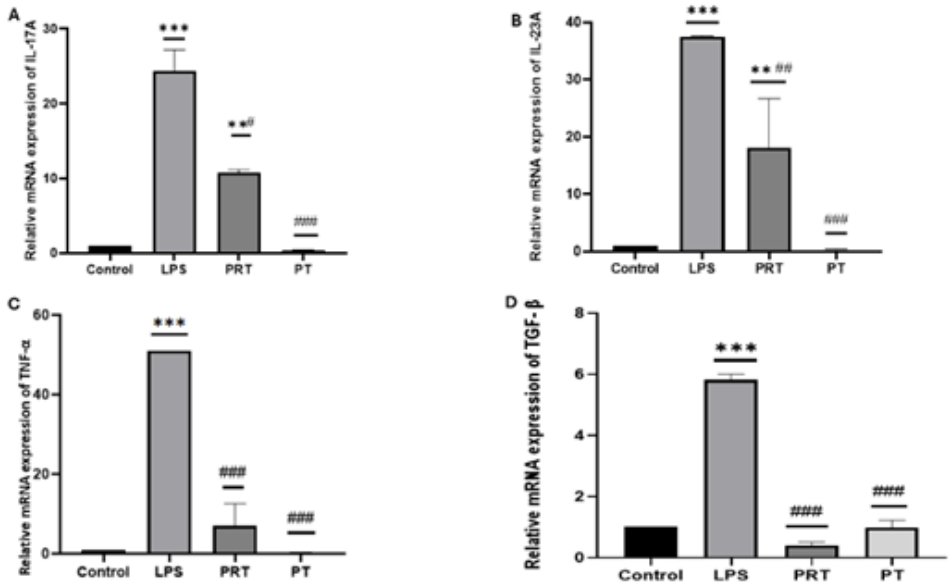

Figure 1 - Estimation of creatinine & BUN: The biochemical estimation of creatinine &BUN in mice kidney were analysed by semi auto analyser. The values were considered statistically significant when *$p < 0.05$and* $p < 0.001$.**

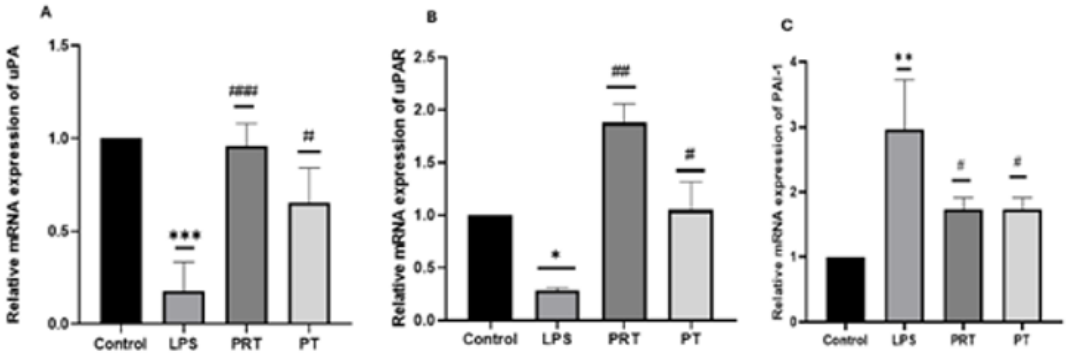

Figure 2. Expression levels of pro-inflammatory cytokines in acute kidney injury.

C57BL/6 mice were exposed to LPS (10 mg/kg) via intraperitoneal administration for 24 hours. Experimental groups include LPS, pre-treatment, and post-treatment groups. In the pretreatment group, thymoquinone (TQ, 18 mg/kg) was administered intraperitoneally for 24 or 48 hours prior to LPS exposure. In the post-treatment group, TQ was administered intraperitoneally 42 or 72 hours following LPS induction. The figure represents the gene expressions of a) IL-17A b) IL-23A c) TNF-ά d) TGF-β.Relative expression of mRNA was normalized with β-actin,p<0.1*** p < 0.01 compared to control; ###p < 0.001, compared to LPS exposure (Mean ± SD, n = 3).

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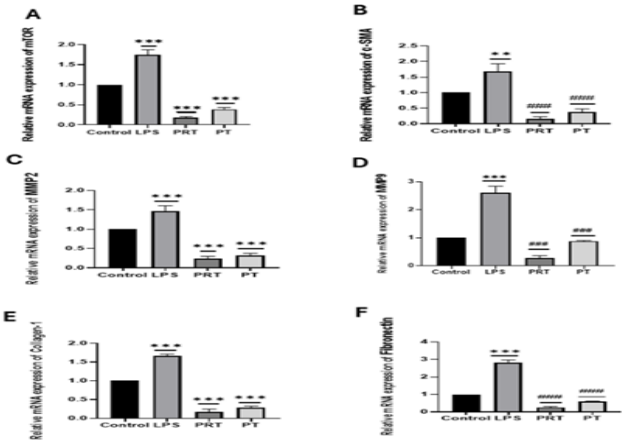
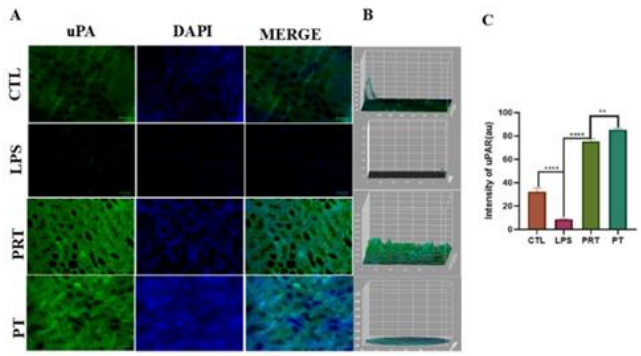


Figure 3. Expression levels of fibrinolytic system in acute kidney injury.

C57BL/6 mice were exposed to LPS (10 mg/kg) via intraperitoneal administration for 24 hours. The experimental groups included LPS, pretreatment, and post-treatment groups. In the pretreatment group, thymoquinone (TQ, 18 mg/kg) was administered intraperitoneally for 24 or 48 hours before LPS exposure. In the post-treatment group, TQ was administered intraperitoneally 42 or 72 hours after LPS induction. The figure represents gene expression of a) uPA and b) uPAR whereas the c) PAI-I expression .The relative mRNA expression was normalized to β-actin. ***p < 0.001 indicates significance compared to the control group, and ###p < 0.001 indicates significance compared to LPS exposure (Mean ± SD, n = 3).

Figure 4. Expression levels of ECM remodelling markers in acute kidney injury.

C57BL/6 mice were exposed to LPS (10 mg/kg) via intraperitoneal administration for 24 hours. The experimental groups included LPS, pretreatment, and post-treatment groups. In the pretreatment group, thymoquinone (TQ, 18 mg/kg) was administered intraperitoneally for 24 or 48 hours before LPS exposure. In the post-treatment group, TQ was administered intraperitoneally 42 or 72 hours after LPS induction. The figure represents expressions of a) mTOR b) α-SMA c) MMP2 d) MMP9 e) Collagen-1 f) Fibronectin .The mRNA expression levels were normalized using β-actin as a reference. ***p < 0.01 denotes a significant difference compared to the control group, while ###p < 0.001 indicates a significant difference compared to LPS exposure (Mean ± SD, n = 3).



A)

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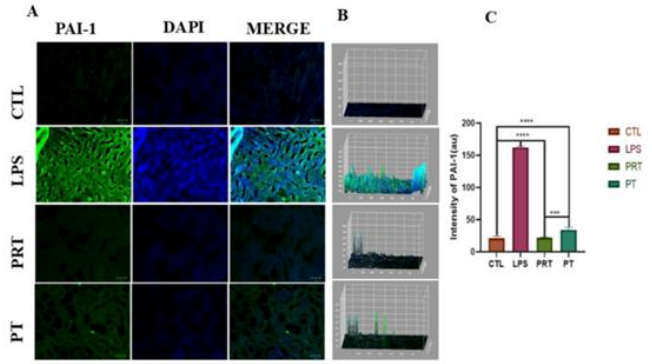

B)

Figure 5: Immunofluorescence Analysis of the Fibrinolytic System in a Thymoquinone-Treated LPS-Induced Acute Kidney Injury Mouse Model.

C57BL/6 mice were administered LPS (10 mg/kg) intraperitoneally for 24 hours. The study included three experimental groups: LPS, pretreatment, and post-treatment. In the pretreatment group, thymoquinone (TQ, 18 mg/kg) was given intraperitoneally for 24 or 48 hours prior to LPS administration. In the post-treatment group, TQ was administered intraperitoneally 42 or 72 hours following LPS exposure.The figure representsProtein expression of a) PAI-I b) uPA (n= 8 per group)visualised by IF staining ,Scale bars: 40µm. **(A,B,C) represents immunofluorescence image , surface plots and bar graphs representing the intensity of fluorescence expressed by proteins uPA and PAI-I**Quantification of fluorescence intensity of uPA , PAI-I using ImageJ . Statistical analysis: One-way ANOVA, multiple comparisons: not significant *(P<0.05),**** *p<0.0001)*.

4. Discussion

The present findings suggest that renal tubular PAI-1 contributes to epithelial differentiation, plasticity, apoptosis, and renal tubular dysfunction. PAI-1 expression plays an important role in several pathological processes, including epithelial dedifferentiation, G2/M proliferative arrest, apoptosis, renal tubular dysfunction accumulation, and renal scarring. It promotes tubular dysfunction, upregulates p53, and activates the TGF-βRI-SMAD3 signaling axis 25. The induction of PAI-1 in cultured cells has been shown to facilitate the progression of chronic inflammatory renal diseases 26. Radiation-induced upregulation of PAI-1 is associated with cellular oxidative stress in renal tubular cells and elevated PAI-1 levels in renal diseases contribute to renal fibrosis and eventual renal failure 27,28 . PAI-1 is also an important regulator of tumor progression 29 .

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that is involved in tissue injury and repair. TGF-β receptors are present in nearly all cells of the body, and its effects are most evident in cellular responses to environmental changes. TGF-β plays a critical role in cell proliferation, ECM production, and fibroblast activity. In the kidney, TGF-β promotes tubule epithelial cell hypertrophy and regulates the production of key ECM components, including collagens, fibronectin, tenascin, proteoglycans, and integrins. TGF-β also prevents the breakdown of newly synthesized ECM by upregulating protease inhibitors and downregulating matrix-degrading enzymes like stromelysin and collagenase 30 .

Studies show that uPAR deficiency in animal models protects against ischemia-reperfusion (IR) injury, reducing renal dysfunction and apoptosis, which are key features of IR injury. In kidney tissue, uPAR deficiency reduces reactive oxygen species (ROS) production and significantly mitigates apoptosis in both IR injury and acute kidney allograft rejection models 31. The uPA/uPAR system not only regulates pericellular

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fibrinolytic and proteolytic activities but also modulates cell proliferation, adhesion, migration, and differentiation 31,32.

IL-17A production in the kidney, primarily by γδ T cells, is induced by IL-23 and contributes to neutrophil recruitment, playing a role in the immune pathogenesis of crescentic glomerulonephritis (GN) 33. IL-17F also plays a critical role in renal tissue injury in experimental GN, making it a potential target for anti–IL-17 cytokine therapies in TH17-mediated autoimmune diseases 34. IL-17 targeting may offer improved diagnostic and therapeutic strategies for AKI (AKI) 34. Elevated IL-17 levels have been observed in renal tissues of patients with primary nephrotic syndrome, where it induces podocyte apoptosis via the Fas/FasL/caspase 8/caspase 3 pathway in an NF-κB-dependent manner 35. In an allogeneic transplantation mouse model, IL-23 exacerbates tissue injury and promotes an anti-donor humoral response 36.

LPS and intravenous TNF exposure induce renal damage, resulting in Ultrastructural changes in glomerular endothelial fenestrations and altered glomerular basement membrane components, contributing to increased albumin permeability and decreased GFR 37. TNF receptor binding induces renal cell apoptosis and cell death in response to ischemia-reperfusion injury 38. TNF receptor levels in circulation predict mortality in patients with end-stage renal disease undergoing dialysis 39. TNF-α mediates inflammatory injury in cisplatin-induced acute renal failure, inducing the expression of adhesion molecules like intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), and P-selectin in endothelial cells 40,41.

In models of renal ischemia-reperfusion injury (IRI), thymoquinone (TQ) has been shown to improve hemodynamic parameters, renal function, and the expression of kidney injury markers and pro-inflammatory cytokines, suggesting its renoprotective potential. TQ reduces the expression of key molecules such as TNF-α, TGF-β1, PAI-1, KIM-1, and NGAL, indicating its anti-inflammatory and anti-fibrotic properties. TQ also acts as an antioxidant and anti-apoptotic agent, inhibiting the NF-κB, PAI-1, caspase-3, and p53 signaling pathways in models of nephrotoxicity induced by cyclosporine A 42,43 . Furthermore, TQ protects against kidney injury caused by various xenobiotics, including chemotherapeutic agents, heavy metals, pesticides, and ischemic shock. The protective mechanisms of TQ involve antioxidation, anti-inflammation, anti-apoptosis, and antifibrosis, with regulation of the antioxidant defense system, NF-κB signaling, caspase pathways, and TGF-β signaling 44. TQ has also been shown to reduce kidney damage by suppressing inflammation, endoplasmic reticulum stress, and apoptosis pathways 45. Additionally, TQ demonstrates significant therapeutic effects on renal cell carcinoma, renal fibrosis, and urinary calculi by scavenging reactive oxygen species (ROS), enhancing antioxidant activity, reducing inflammatory mediators, inducing apoptosis, and inhibiting cell migration and invasion 46. TQ inhibited the increased expression of collagen-I, α-SMA, and TIMP-1 induced by thioacetamide (TAA) 47. The inhibitory effect of TQ on Neuro-2a cell migration was mediated by the suppression of MMP-2 and MMP-9 expression 48.

5. Acknowledgement

We sincerely acknowledge Yenepoya Research Centre and Yenepoya (Deemed to be University) for their support through the seed grant no :YU/Seed grant/083-2019, which has significantly contributed to the progress of this research. The authors extend their sincere gratitude to Dr. Ramdas Nayak and Professor Yashodhar P. Bhandary for their invaluable guidance, mentorship, and support throughout the course of this study. We also acknowledge the collaborative efforts of all co-authors for their significant contributions to the research and manuscript preparation.

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