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Vitexin alleviates renal ischemia/reperfusion injury in rats via activation of Nrf2 and inhibition of necroptosis pathways

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Abstract

Background *Renal ischemia/reperfusion injury is a frequent kidney condition caused by various conditions like kidney and heart surgery. Renal ischemia causes cellular damage and death, whereas reperfusion reintroduces blood into the kidney but generates inflammatory reactions and cellular damage, worsening kidney injury. Vitexin is present in the leaves and other various parts of several medicinal plants. It has excellent safety and pleiotropic effects in oxidation, inflammation, cancer, infection, and neuro-cardio-hepatoprotection. According to our knowledge, there is limited data about the nephroprotective effects of vitexin on renal ischemia/reperfusion injury-induced acute kidney injury. Therefore, this study seeks to assess the nephroprotective effects of vitexin through Nrf2 and RIPK1/MLKL molecular pathways.*

Materials and methods *Twenty-four Sprague Dawley rats were allocated into four groups: The sham group was subjected to laparotomy without the I/R induction. The I/R, I/R+Veh, and I/R+VTN groups were exposed to 30 minutes of bilateral renal ischemia, which was followed by 24 hours of reperfusion. The I/R+Veh and I/R+VTN groups administered DMSO and 40 mg/kg of vitexin by the I. P. route one hour before the I/R induction, respectively. Renal damage marker (KIM-1), inflammatory markers (TNF-α, IL-1β, and NF-κB), and apoptotic marker (caspase-3) were measured by ELISA. Nrf2 and MLKL were assessed by IHC, and RIPK1 was analyzed by RT-qPCR, in addition to histopathological examination.*

Results *The control and vehicle groups showed substantial elevation of KIM-1, NF-κB, TNF-α, IL-1β, caspase-3, RIPK1, MLKL, and histopathological findings. Conversely, vitexin displayed a remarkable reduction in renal damage, inflammatory markers, cellular death, and histopathological findings. In the vitexin-treated group, nuclear translocation of Nrf2 was markedly escalated. Moreover, molecular docking showed that vitexin interacted with Keap1.*

Conclusion *The present study confirmed that vitexin had substantial nephroprotective effects against renal IRI. Vitexin diminished inflammation, necroptosis, apoptosis, and necrosis through activation of Nrf2 and inhibition of RIPK1/MLKL pathways.*

Keywords *Vitexin, renal ischemia/reperfusion injury, Nrf2, necroptosis, RIPK1, MLKL.*

1 INTRODUCTION

Kidneys are bean-like excretory organs placed in the retroperitoneum on either side of the spinal column in the back of the abdomen. Both kidneys weigh about 0.8% of the body weight. Despite this low weight, 25% of the cardiac output reaches the kidneys [1]. Due to their high mitochondrial number and oxygen expenditure, the kidneys have the second-highest resting metabolic rate after the heart. They use most of their energy for reabsorption and secretion of nutrients/electrolytes and waste products [2]. Renal ischemia/reperfusion injury (IRI) is a frequent kidney condition caused by various conditions like kidney and heart surgery. Renal ischemia causes cellular damage and death due to metabolite buildup and oxygen deficiency. In contrast, reperfusion reintroduces blood into the kidney but generates inflammation and cell damage, worsening kidney injury [3].

Nuclear factor erythroid 2-related factor 2 (Nrf2) governs the expression of proteins involved in cellular responses to oxidation. It is negatively controlled by kelch-like ECH-associated protein 1 (Keap1), a protein that interacts with Nrf2 in the cytoplasm to promote its polyubiquitination and subsequent degradation.

Constitutive Nrf2 degradation facilitates low basal levels for proteasomal degradation [4]. Nrf2 belongs to a cap'n'collar family of basic region-leucine zipper (bZIP) proteins. This nuclear factor consists of seven Nrf2-ECH homology domains [5].

Cellular demise can be classified into two groups: programmed, like apoptosis, and non-programmed, like necrosis [6]. Necroptosis was first mentioned in 2005 as a regulated type of necrosis. It's caspase-dependent and pro-inflammatory. Additionally, it displays characteristics of both apoptosis and necrosis. It resembles apoptosis in that it is tightly controlled and initiated by specific signals. While, it is morphologically related to necrosis, where the cells are ruptured, releasing damage-associated molecular patterns and inflammation [7,8]. Stressful conditions like IRI can activate necroptosis through many ligand/receptor signaling pathways, among which the tumor necrotic factor-alpha (TNF-α)/TNF receptor 1 interaction is the most studied [9].

Vitexin is present in the leaves and other parts of several medicinal plants, including hawthorn, where its leaves are enriched with Vitexin. It has excellent safety and pleiotropic effects in oxidation, inflammation, cancer, infection, and neuro-cardio-hepatoprotection [10]. Vitexin has a very low oral bioavailability; the intestine degrades about 94% of it, whereas the liver and stomach metabolize about 5% and 30%, respectively. However, it is distributed widely and rapidly in different tissues and excreted by bile and kidney [11]. The acute or subacute oral intake of vitexin by rats of both sexes has seemed to be well tolerated, with insignificant toxicity to the nervous and respiratory systems or other physiological processes [12]. According to our knowledge, there is limited data about the nephroprotective effects of vitexin on renal IRI-induced acute kidney injury (AKI). Therefore, this study seeks to assess the nephroprotective effects of vitexin through Nrf2 and RIPK1/MLKL molecular pathways.

2 MATERIALS AND METHODS

2.1 Chemicals and assay kits

Vitexin (CAS No.: 3681-93-4, MW: 432.38, purity: ≥95%) was purchased from Macklin Biochemical Technology, China. Dimethyl sulfoxide (DMSO) (CAS No.: 67-68-5, MW: 78.13) was purchased from CDH, India. Ketamine (10%) and xylazine (2%) were purchased from Alfasan, Netherlands. Rat ELISA kits for kidney injury molecule-1 (KIM-1) (Cat. No. SL0433Ra), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Cat. No. SL0537Ra), TNF-α (Cat. No. SL0722Ra), Interleukin-1 beta (IL-1β) (Cat. No. SL0402Ra), and caspase-3 (Cat. No. SL0152Ra) were purchased from SUNLONG, China. Nrf2 (Cat. No. E-AB-93081) and mixed lineage kinase domain like pseudokinase (MLKL) (Cat. No. E-AB-67102) polyclonal antibody immunohistochemistry (IHC) kits were purchased from ELABSCINE, China.

GoTaq® 1-Step RT-qPCR system (Cat. No. A6020), AddScript cDNA synthesis kit (Cat. No. 22701), receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers, and Easy-spin™ (DNA-free) total RNA extraction kit (Cat. No. 17221) were purchased from Promega/USA, Addbio/Korea, Macrogen/Korea, and Intron/Korea, respectively.

2.2 Animals

The resource equation method [13] was utilized for sample size calculation, as estimating the standard deviation and effect size was challenging. Since the study included four groups, the total number of animals ranged from 16 to 24. Therefore, 24 animals were used. Twenty-four Sprague Dawley rats, about eight weeks of age, and 200-250 grams of weight were used in the current study. They were obtained from the Faculty of Sciences, University of Kufa, Najaf, Iraq. Rats were kept in separate cages, with water and diet *ad libitum*, under-regulated environments of a temperature of $25 \pm 2^{\circ}C$ and a 12-hour of light and dark. After the surgery, each rat was kept in a cage until the sample collection. After a two-week adaptation period, rats were used in the study. The Central Committee of Bioethics at the University of Kufa approved all the procedures and experimental design (Approval No. 20554) on August 29, 2024.

2.3 Experimental design

Randomly, animals were assigned into four groups (six rats each):

A. Sham group: Rats were anesthetized and underwent laparotomy without ischemia/reperfusion (I/R).

B. I/R group: Rats were anesthetized and subjected to 30 minutes of bilateral renal ischemia, then 24 hours of reperfusion.
C. I/R+Vehicle group (I/R+Veh): This group likes the I/R group, but the solvent of vitexin (10% DMSO) was administered to the animals by intraperitoneal (I.P.) route one hour before bilateral renal ischemia.
D. I/R+Vitexin (I/R+VTN) group: This group likes the I/R group, but 40 mg/kg of vitexin was administered to the animals [14] by the I.P. route one hour before bilateral renal ischemia.

2.4 Model of Renal Ischemia/reperfusion Injury

Renal IRI model was performed based on several scientific studies [15–18]. Under aseptic conditions, rats were anesthetized with a combination of I.P. 100 mg of ketamine and 10 mg of xylazine for each kg of the rat weight [19,20]. The extent of anesthesia is examined by the tail pinch and pedal reflex. Afterward, rats were placed supine by fixing their limbs with surgical plaster. A midline laparotomy was made, the intestine was retracted, and the renal pedicle was exposed. Bilateral ischemia was made by clamping the renal blood vessels with non-traumatic microvascular clamps for 30 minutes; the ischemic phase is examined visually by changing the kidneys' color. To keep rats well hydrated, 1ml of normal saline 0.9% was injected into the abdomen, then covered with wet warm gauze. After 30 minutes of ischemia, the clamps were removed, and the reperfusion phase started, which was examined visually by changing the kidneys' color. The abdominal incision was sutured in two layers with zero nylon surgical sutures, disinfected with 10% povidone-iodine, and covered with surgical plaster. The time for the reperfusion phase was 24 hours; afterward, rats were anesthetized again for tissue and blood collection. Lastly, rat euthanasia was done with a high dose of anesthesia [21].

2.5 Tissue collection

At the end of the 24-hour reperfusion phase, both kidneys were harvested. The left kidney was divided into two halves, put in an Eppendorf tube, and snap-frozen in liquid nitrogen. One half was used to measure the gene expression of RIPK1 by RT-qPCR, while the other half was used to measure KIM-1, NF-κB, TNF-α, IL-1β, and caspase-3 levels by ELISA technique. The right kidney was fixed in 10% formalin and stored until used for measuring Nrf2 and MLKL by IHC technique and for hematoxylin and eosin (H&E) staining.

2.6 Tissue preparation

For ELISA and RT-qPCR, tissue was prepared by thawing the kidney and cleaning it with phosphate buffer saline (PBS). Then, the kidney was weighed and homogenized with 1:10 PBS comprising 1% Triton 100X with a cocktail of protease inhibitors. More homogenization was done by using a high-intensity ultrasonic liquid processor to obtain the supernatant, which is used to measure the level of KIM-1, NF-κB, TNF-α, IL-1β, and caspase-3 in tissue by ELISA and RIPK1 by RT-qPCR.

2.7 Docking study

BTB domain of Keap1 was acquired from the protein data bank (PDB ID: 4CXI). The protein preparation, performed by MOE software 2015, involved eliminating aqueous solvent molecules to enhance the receptor/ligand interactions and adding protons to enhance protein preparation. The two-dimensional structure of vitexin was designated by Chem-Bio Draw pro19.0. MOE was used to protonate the three-dimensional structure, add the partial charge, and minimize the energy. Vitexin was docked, and the pose exhibiting a higher S-score with an appropriate RMSD value was chosen.

2.8 Renal Histopathology

The formalin-fixed kidney was processed through various tissue processing steps using an automated tissue processor. A microtome was used to obtain 5-um sections of paraffin blocks, which were mounted on slides for tissue staining. The slides were then de-paraffinized, rehydrated, washed, and stained with H&E. An independent histopathologist blinded to experimental groups examined the histopathologic changes. The degree of tubular damage was scored from 0 to 4, where 0 indicates no damage, 1 signifies less than 25%, 2 represents 25-50%, 3 denotes 50-75%, and 4 exceeds 75% [22].

2.9 IHC staining

The slides were de-waxed with xylene, rehydrated by immersing them in descending concentrations of ethanol, and rinsed with distilled water. The slides were incubated with the retrieval solution in a water bath, cooled at room temperature, and rinsed with the washing buffer solution to unmask the sample antigens. Peroxidase, a blocking agent, prevented non-specific binding. The post-protein block was added for five minutes and washed again. The primary antibody against Nrf2 and MLKL were diluted to 1:100, added to the slides, incubated, and washed with buffer solution. Consequently, the secondary antibody was added to the slides. The next steps include adding horseradish peroxidase, chromogen, and hematoxylin (the counter stain).

As in H&E staining, levels of Nrf2 and MLKL were examined by an independent histopathologist blinded to study groups. The quick H-score is measured as in Charafe-Jauffret et al [23] and Parris et al [24].

2.10 Gene expression

Total RNA extraction was done by Easy-spin™ kit. Subsequently, cDNA was generated using the AddScript cDNA Synthesis Kit. Primers (Table 1) were made following the instructions of the manufacturing company. They were reconstituted with double distilled water to a stock solution of 100 pM/µl, which was stored at -20 °C. A 10 pM/µl concentration was produced as a working primer. Lastly, the gene expression assay was performed using the GoTaq® RT-qPCR System protocol. The $2^{-\Delta\Delta CT}$ technique was employed to examine the gene expression data.

Table 1. Primers' sequence of RIPK1 and GAPDH.

Gene	5'-"	Product'(bp)	Accession number
RIPK1			
Forward	GACCGAGTTCACAACCACCA	75	XM_017600528.3
Reverse	TGTTAGCGAAGACGGCTTGA	75	XM_017600528.3
GAPDH			
Forward	ATGACTCTACCCACGGCAAG	89	NM_017008
Reverse	CTGGAAGATGGTGATGGGTT	89	NM_017008

2.11 Statistical Analysis

The normality test was done using the Shapiro-Wilk test. For parametric data, the one-way analysis of variance (ANOVA) was applied to check the significant differences among study groups, and multiple comparisons were performed using the Tukey post-hoc test. Data was presented as mean±SD. GraphPad Prism 10 was used for data visualization and statistical analysis.

3 RESULTS

3.1 Vitexin reduces the renal damage marker in the renal IRI

The KIM-1 level was measured to study the effect of vitexin on renal functions. The induction of the IRI model, as in the I/R and I/R+Veh groups, substantially elevated the KIM-1 level relative to the sham group ($p<0.0001$). On the contrary, vitexin markedly reduced the KIM-1 level in contrast to the induction and vehicle groups (I/R ($p<0.01$) and I/R+Veh ($p<0.0001$) groups). However, a non-statistical variation was experienced between I/R and I/R+Veh groups ($p>0.05$) (Figure 1).

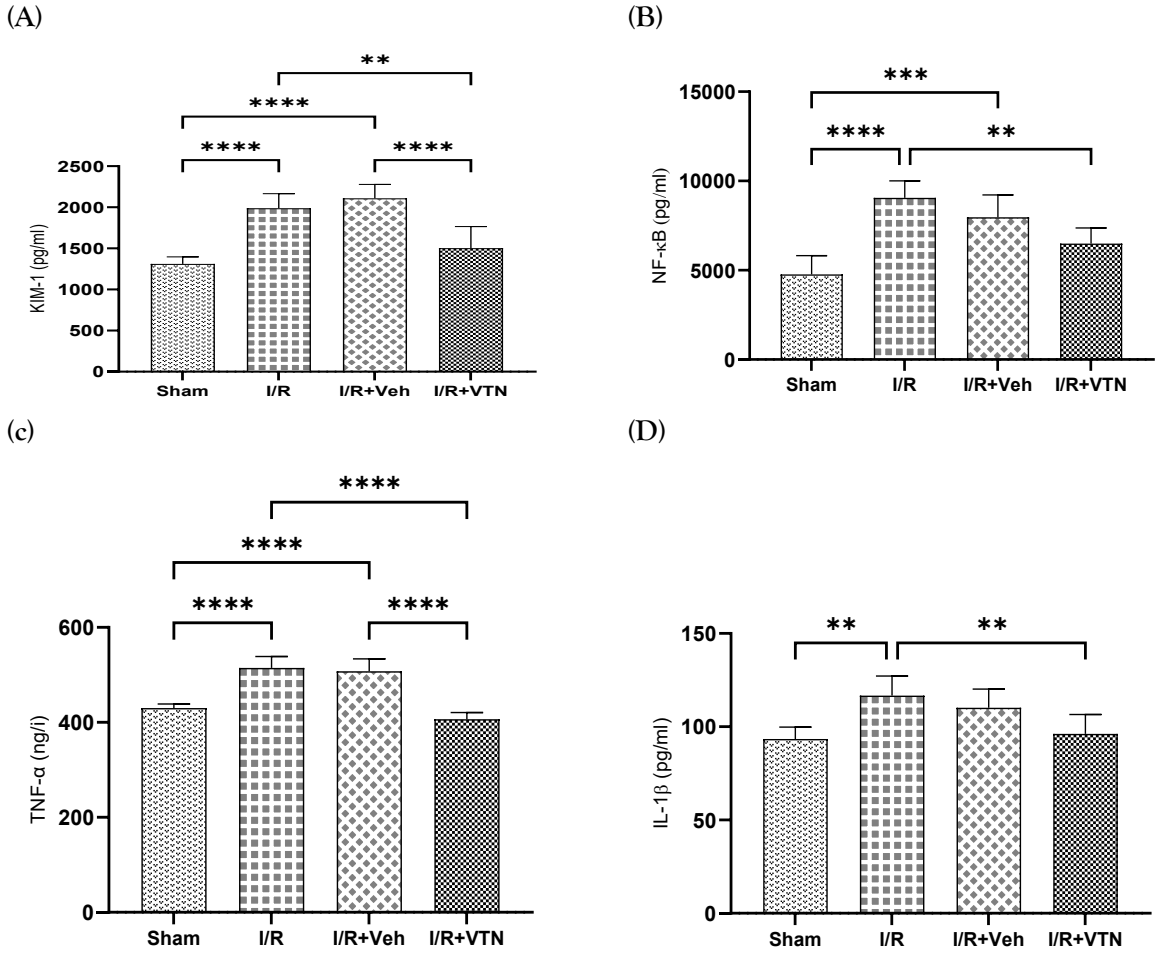
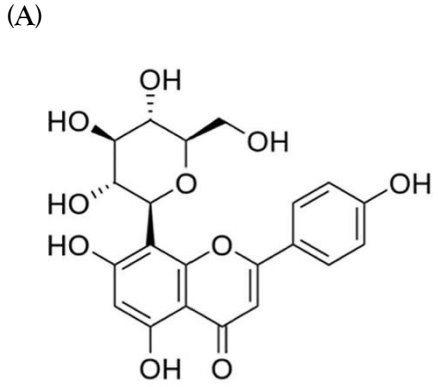


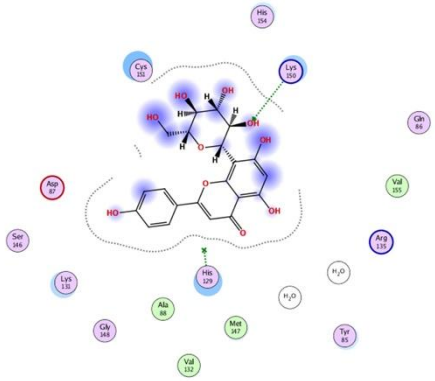
Figure 1. Effects of vitexin on renal damage and inflammatory markers. **(A)** KIM-1. **(B)** NF-κB. **(C)** TNF-α. **(D)** IL-1β. The sham group was subjected to laparotomy without the I/R induction. The I/R, I/R+Veh, and I/R+VTN groups were exposed to 30 minutes of bilateral renal ischemia, which was followed by 24 hours of reperfusion. The I/R+Veh and I/R+VTN groups administered the vehicle and 40 mg/kg of vitexin by the I.P. route one hour before the I/R induction, respectively.KIM-1, NF-κB, TNF-α, and IL-1β were measured by ELISA in renal tissue. Data expressed as mean ± SD. *($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$).

3.2 Vitexin interacts with Keap1

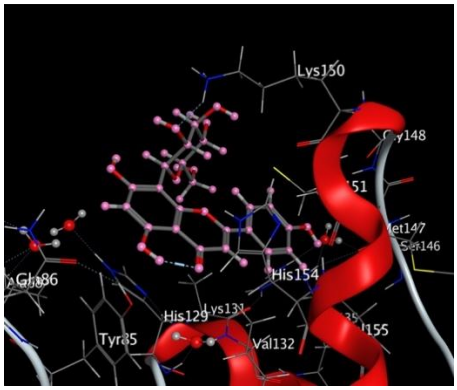
The in-silico molecular docking study showed that vitexin binds with Keap1 through Lys150 (Figure 2A-C).



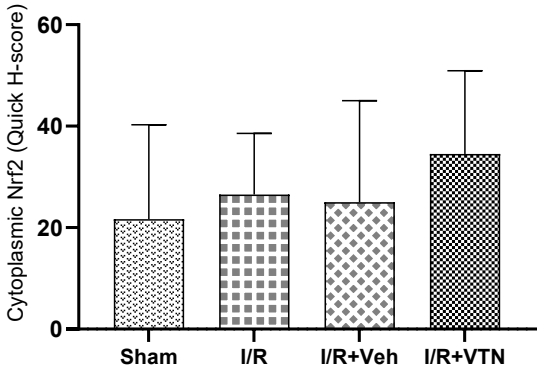
(B)



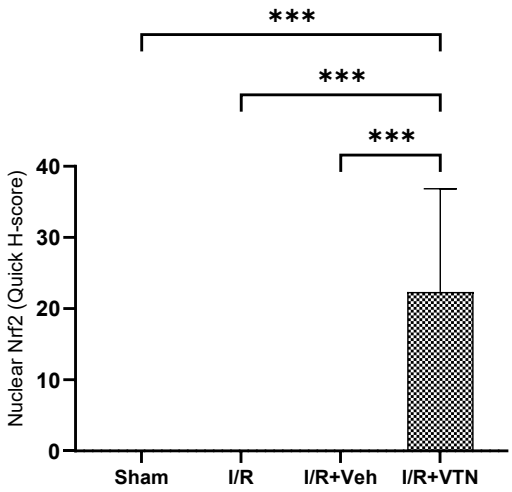
(C)


Lys150
His154
His129
Tyr85
Val132

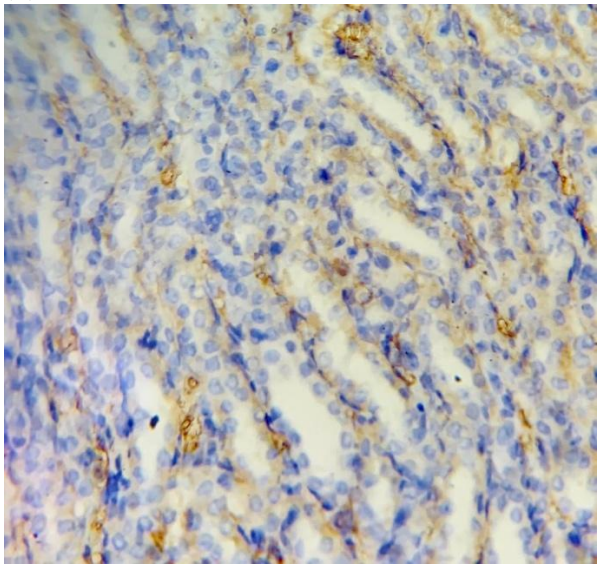
(D)


Cytoplasmic Nrf2 (Quick H-score)
60
40
20
0
Sham
I/R
I/R+Veh
I/R+VTN

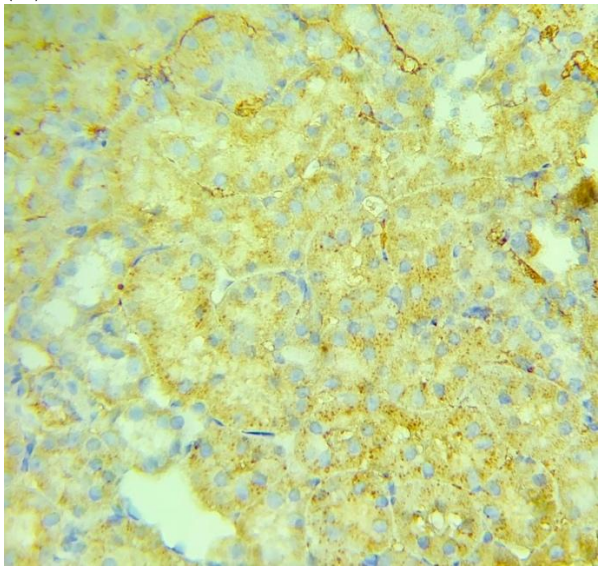
(E)


***
***
***
Nuclear Nrf2 (Quick H-score)
40
30
20
10
0
Sham
I/R
I/R+Veh
I/R+VTN

(F)



(G)



(H)

(I)

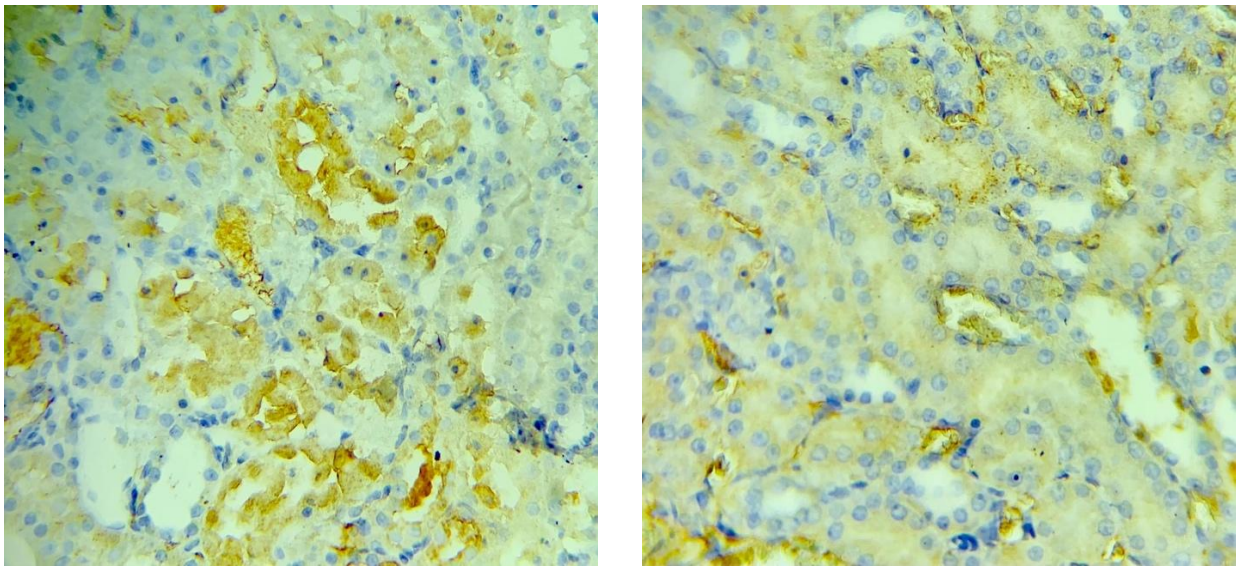


Figure 2. Molecular docking of vitexin with Keap1. **(A)** Chemical structure of vitexin. **(B)** The 2D model of molecular docking of the vitexin-Keap1 complex. **(C)** The 3D model of molecular docking of the vitexin-Keap1 complex. **(D)** The quick H-score of cytoplasmic Nrf2. **(E)** The quick H-score of nuclear Nrf2. **(F)** Immunohistochemical detection of Nrf2 in the sham group. **(G)** Immunohistochemical detection of Nrf2 in the I/R group. **(H)** Immunohistochemical detection of Nrf2 in the I/R+Veh group. **(I)** Immunohistochemical detection of Nrf2 in the I/R+VTN group. The sham group was subjected to laparotomy without the I/R induction. The I/R, I/R+Veh, and I/R+VTN groups were exposed to 30 minutes of bilateral renal ischemia, which was followed by 24 hours of reperfusion. The I/R+Veh and I/R+VTN groups administered the vehicle and 40 mg/kg of vitexin by the I.P. route one hour before the I/R induction, respectively. Molecular docking was performed by the MOE software in 201. The Quick H-score was determined through the multiplication of the immunostaining intensity by the percentage of immunostained cells. Cytoplasmic and nuclear Nrf2 were measured by IHC staining in renal tissue. S = -5.68647, RMSD = 1.596419. Data expressed as mean ± SD. ****($p<0.0001$).

3.3 Vitexin increases the nuclear localization of Nrf2

To study the role of vitexin on Nrf2, cytoplasmic and nuclear Nrf2 were measured. No change in cytoplasmic Nrf2 was seen among the tested groups ($p>0.05$). On the other hand, the group that received vitexin showed a marked elevation in nuclear Nrf2 as opposed to the other groups ($p<0.001$) (Figure 2).

3.4 Vitexin reduces inflammation in the renal IRI

NF-κB, TNF-α, and IL-1β levels were measured in renal tissue. There was a substantial increment in the I/R group in contrast to the sham group for NF-κB ($p<0.0001$), TNF-α ($p<0.0001$), and IL-1β ($p<0.01$). Moreover, the vehicle group was markedly elevated relative to the sham group for NF-κB ($p<0.001$) and TNF-α ($p<0.0001$). In contrast, vitexin notably reduced the levels of these markers relative to I/R and I/R+Veh groups. In detail, for NF-κB ($p<0.01$), TNF-α ($p<0.0001$ vs. I/R, and I/R+Veh), and IL-1β ($p<0.01$). Nevertheless, there was insignificant variation between the IRI induction and vehicle groups for all the studied markers ($p>0.05$) (Figure 1).

3.5 Vitexin downregulates the expression of necroptotic pathway markers in the renal IRI

To study the effects of vitexin on necroptosis, RIPK1 and MLKL were measured. The gene expression of RIPK1 in the I/R and I/R+Veh groups was drastically elevated in contrast to the sham group ($p<0.0001$). Conversely, the treatment with vitexin substantially downregulated the level of RIPK1 in the I/R+VTN group against I/R and I/R+Veh groups ($p<0.0001$). In the same vein, MLKL levels in the I/R and vehicle groups were considerably lower than in the sham and vitexin-treated groups ($p<0.0001$). However, the RIPK1 and MLKL levels in the I/R group showed no substantial difference with the vehicle group ($p>0.05$) (Figure 3).

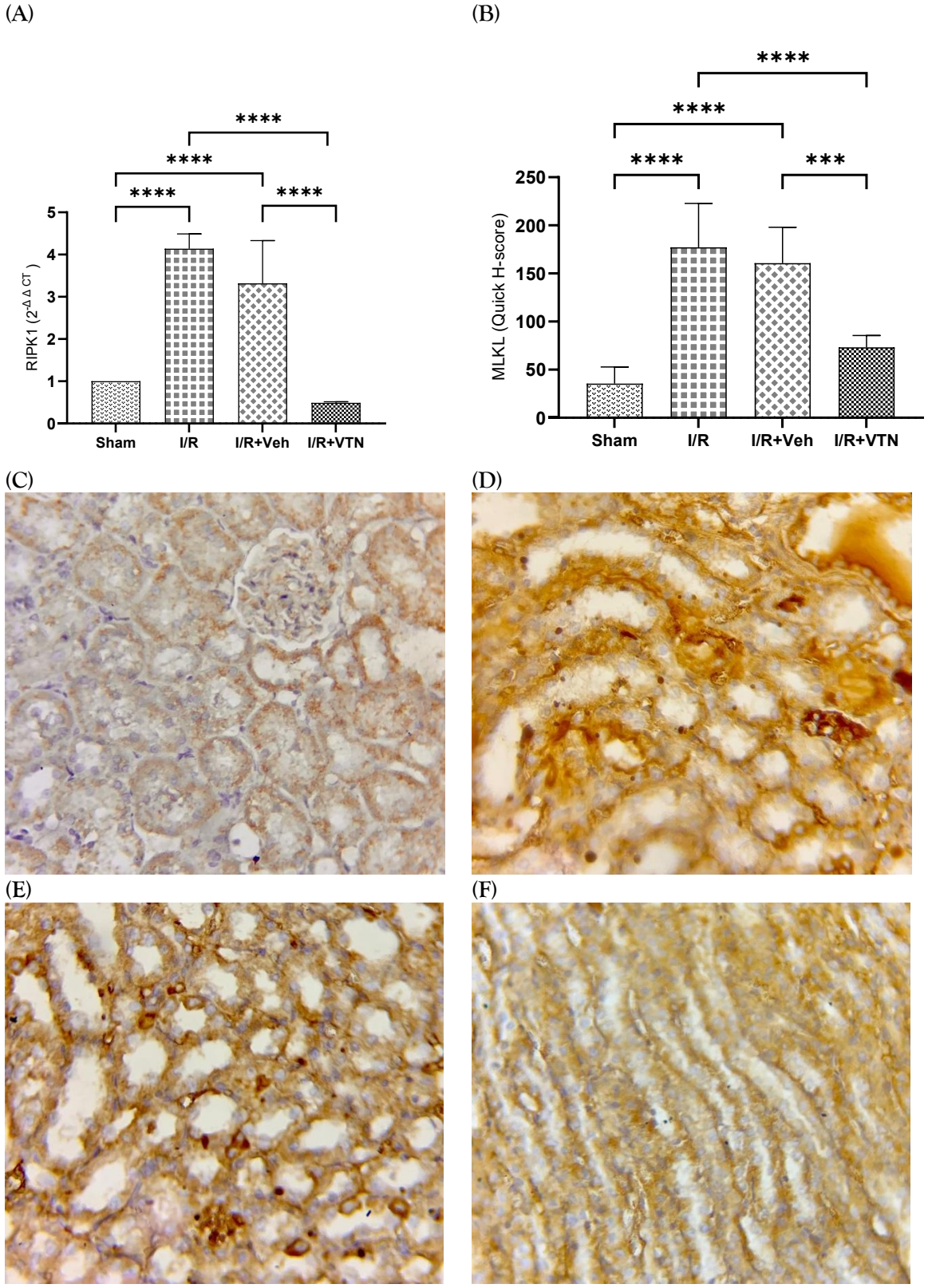
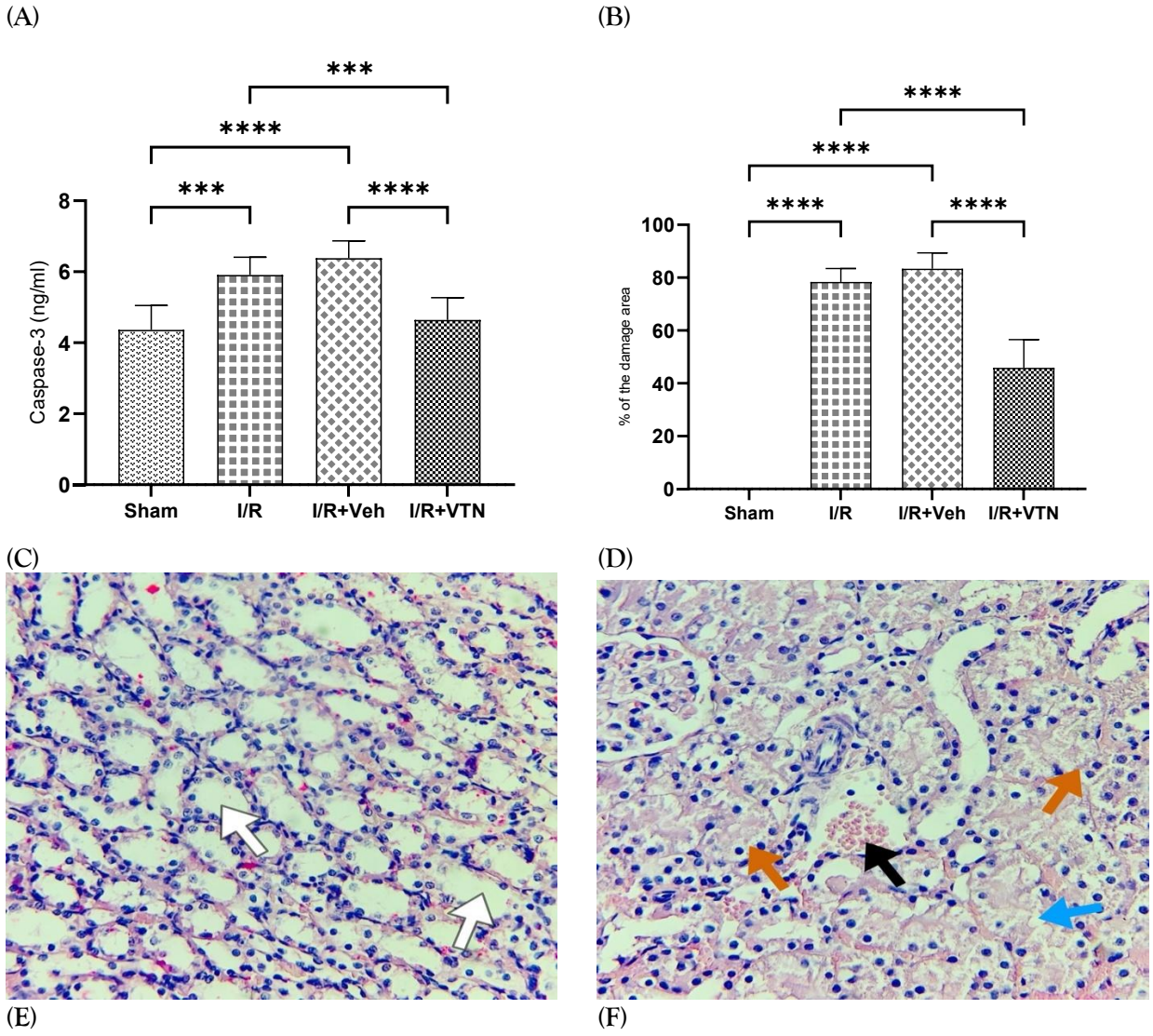


Figure 3. Effects of vitexin on the necroptotic pathway markers. **(A)** Fold change of RIPK1. **(B)** The quick H-score of MLKL. **(C)** Immunohistochemical detection of MLKL in the sham group. **(D)** Immunohistochemical detection of MLKL in the I/R group. **(E)** Immunohistochemical detection of MLKL in the I/R+Veh group.

(F) Immunohistochemical detection of MLKL in the I/R+VTN group. The sham group was subjected to laparotomy without the I/R induction. The I/R, I/R+Veh, and I/R+VTN groups were exposed to 30 minutes of bilateral renal ischemia, which was followed by 24 hours of reperfusion. The I/R+Veh and I/R+VTN groups administered the vehicle and 40 mg/kg of vitexin by the I.P. route one hour before the I/R induction, respectively. RIPK1 fold change was measured by RT-qPCR in renal tissue, and MLKL was measured by IHC in renal tissue. The Quick H-score was determined through the multiplication of the immunostaining intensity by the percentage of immunostained cells. Data expressed as mean ± SD. ***($p<0.001$), ****($p<0.0001$).

3.6 Vitexin mitigates apoptosis in the renal IRI

The caspase-3 level in renal tissue was measured to assess the effectiveness of vitexin in mitigating apoptosis. The induction of the IRI model in I/R and I/R+Veh groups remarkably escalated the caspase-3 level in contrast to the sham group ($p<0.001$ vs. I/R, $p<0.0001$ vs. I/R+Veh). Inversely, there was a pronounced decline in caspase-3 in the treatment group, opposite to the I/R and vehicle groups ($p<0.001$vs I/R+Veh and $p<0.001$ vs I/R). Nevertheless, the I/R group did not demonstrate any notable variation with the vehicle group ($p>0.05$) (Figure 4).



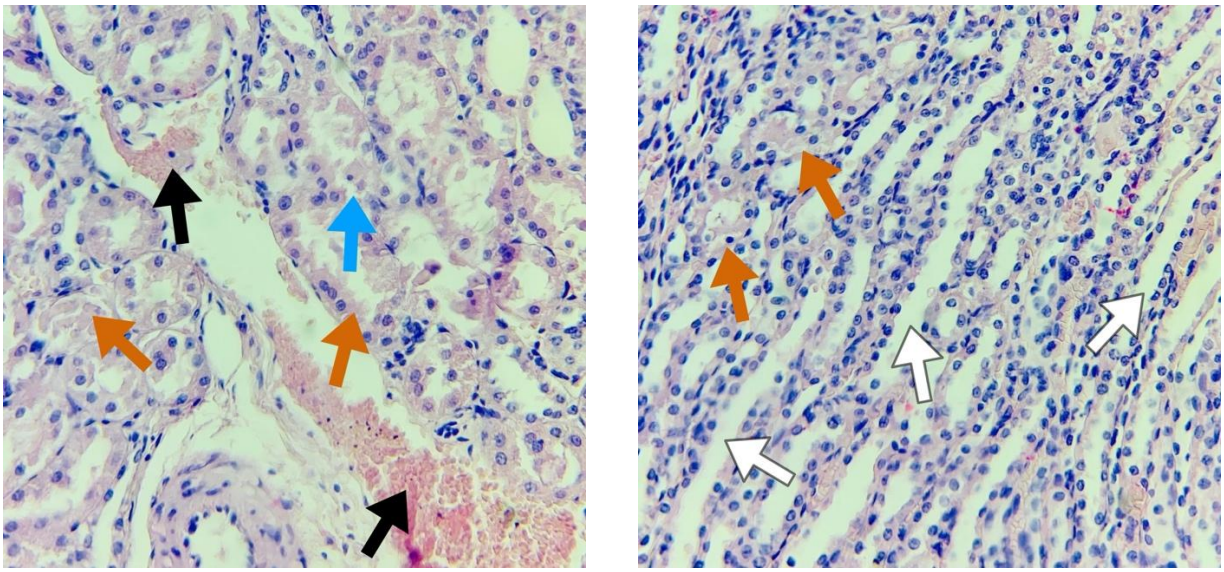


Figure 4. Effects of vitexin on histopathological findings. **(A)** Caspase-3. **(B)** The percent of the damaged area. **(C)** Sham group: Normal histology. **(D)** I/R group: The damage score equals 4, impacting 80% of investigated renal tubules. **(E)** I/R+Veh group: The damage score equals 4, impacting 85% of investigated renal tubules. **(F)** I/R+EGZ group: The damage score equals 2, impacting 35% of investigated renal tubules. The sham group was subjected to laparotomy without the I/R induction. The I/R, I/R+Veh, and I/R+VTN groups were exposed to 30 minutes of bilateral renal ischemia, which was followed by 24 hours of reperfusion. The I/R+Veh and I/R+VTN groups administered the vehicle and 40 mg/kg of vitexin by the I.P. route one hour before the I/R induction, respectively. White arrow (normal glomerulus tubules), blue arrow (cytoplasmic vacuoles), orange arrow (cytoplasmic swelling and increased cytoplasmic eosinophilia), black arrow (vascular congestion and hemorrhage). Caspase-3 was measured using ELISA. Histopathological examinations were prepared using H&E staining in renal tissue. Magnification X400. Bars expressed as mean ± SD. ***($p<0.001$), ****($p<0.0001$).

3.7 Vitexin improved the histopathological findings in the renal IRI

The sham group had a normal histopathological examination (Figure 4C). The I/R group, on the other hand, showed inflammation, edema, cellular swelling, loss of the brush border, and a high cytoplasmic eosinophilia ($p<0.0001$) (Figure 4D). Similar findings were seen in the vehicle group ($p>0.05$) (Figure 4E). The vitexin-treated group showed improved histological features against the I/R and vehicle groups ($p<0.0001$) (Figure 4E).

4 DISCUSSION

AKI causes kidney function to decline rapidly over hours to days. It is typically diagnosed with other acute disorders and is common in severely ill patients. Clinical implications of AKI involve waste products, electrolytes, and fluid accumulation, as well as subtle symptoms like impaired immunity and non-renal organ dysfunction [25]. Renal IRI is a critical and primary cause of AKI. It is a complex condition involving many molecular mechanisms and pathways related to cellular dysfunction and death, such as necroptosis and apoptosis [15,26]. To date, no specific medication has been approved for prevention, treatment, or enhancing AKI recovery [27,28].

Currently, the laboratory assessment of renal impairment relies on conventional indicators that lack sensitivity and specificity, including serum creatinine, urea, and electrolyte concentrations [29]. Numerous rat models of AKI showed that the expression of KIM-1 in the apical side of the proximal tubules is triggered by toxic damage and ischemia. KIM-1 is considered an ideal biomarker for renal damage. Following toxic and ischemic injury, it is synthesized and released, escalating its level in blood and urine [30].

The current study exhibited that the induction of the I/R increased the KIM-1 level, while pretreatment with 40 mg/kg of vitexin extremely reduced the KIM-1 concentration. Ijaz et al. [31] proved that vitexin decreased KIM-1 concentration in a cisplatin-induced model of renal toxicity.

KIM-1 is upregulated in proximal epithelial cells due to IRI and toxic damage, turning the epithelial cells into phagocytic cells, alleviating and repairing the tubular damage, inducing cellular autophagy, and disposing of necrotic tissue and apoptotic cells [32,33].

Nrf2 is an efficient orchestrator that regulates around 200 cytoprotective genes. Because of oxidation or electrophiles, Keap1 encounters a conformational change that separates Nrf2 from Keap1 and translocates it to the nucleus, upregulating the target genes [4,34].

Both cytoplasmic and nuclear Nrf2 were assessed. The current study revealed that the cytoplasmic Nrf2 level did not substantially change between the study groups; despite this, a remarkable elevation of nuclear Nrf2 level was noticed in the vitexin-treated group. Furthermore, to point out the mechanism by which vitexin did its effect, we docked vitexin with Keap1. The in-silico study results confirmed that vitexin binds with Keap1 through Lys150., near the critical Cys151. Additional investigations are required to emphasize the importance of this binding. The results indicated that vitexin increased the nuclear translocation of Nrf2, possibly by affecting the Nrf2-Keap1 complex.

NF-κB works as a transcriptional factor governing various biological mechanisms involving inflammation, tissue damage, and repair [35]. Expression of NF-κB can be provoked by the reperfusion period of renal IRI, leading to induction in the levels of TNF-α and IL-1β [36]. Conversely, Nrf2 activation suppresses the NFκB pathway by enhancing antioxidant genes, causing reduced pro-inflammatory cytokines [37]. Therefore, we measured the NF-κB, TNF-α, and IL-1β levels in renal tissue. The present study displayed that these biomarkers are boosted after the induction of I/R, whereas the administration of vitexin lowered these biomarkers in contrast to the control group. These findings agree with Duan et al. [38].

Numerous molecular pathways are initiated from the interaction of TNF-α with TNFR1, yielding distinct consequences involving cellular survival, apoptosis, and necroptosis. Necrosome is the central complex of necroptosis, consisting of RIPK1, RIPK3, and MLKL [39]. Necroptosis is deemed to have an essential role in the progression of renal IRI [40]. Necroptosis starts when RIPK1 and RIPK3 are phosphorylated and activated, followed by the initiation and recruitment of the RIPK1-RIPK3 complex. Then, MLKLs are phosphorylated and oligomerized. Consequently, clusters of phosphorylated MLKL move to the plasma membrane to initiate necroptosis either by ion channel influx regulation or by forming pores that damage the membrane [41].

Therefore, to evaluate the effects of vitexin on cellular survival and death in the rat model of AKI, at the beginning, we studied the molecular pathway of necroptosis by measuring RIPK1 and MLKL levels in renal tissue. The results demonstrated a dramatic boost in both RIPK1 and MLKL levels in the induction and vehicle groups, while vitexin reduced the level of these markers. In their research, Zhang et al. [42] showed that vitexin markedly lowered the phosphorylated RIPK1 and MLKL in the hepatocyte cell line.

Subsequently, we investigated the apoptosis by measuring caspase-3 in renal tissue. Caspase-3 plays a central role in regulating apoptosis, acting as a key executioner [43]. The current study proved that treatment with vitexin remarkably lowered the caspase-3 level in contrast to the I/R and vehicle groups. Supporting our findings, research by Jiang et al. [44] found that adding vitexin reduced caspase-3 levels in a rat model of ischemic stroke.

The histological analysis reinforces the nephroprotective potential of vitexin, revealing enhanced renal parenchymal integrity and improved tissue architecture in the treated group. Noor et al. [45] showed that vitexin had histoprotective effects on rat liver.

5 CONCLUSION

The present study confirmed that vitexin had substantial nephroprotective effects against renal IRI. Vitexin diminished inflammation, necroptosis, apoptosis, and necrosis through activation of Nrf2 and inhibition of RIPK1/MLKL pathways.

Ethical Considerations

The policy of the University of Kufa for scientific purposes is followed in the care and usage of animals. Pain, distress, and discomfort were avoided or minimized by anesthetizing rats with a mixture of ketamine and xylazine based on their body weight. Upon completion of the procedure, a high dose of anesthesia was used to euthanize the rats. The Central Committee of Bioethics at the University of Kufa approved all the procedures and experimental design (Approval No. 20554) on August 29, 2024.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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Authors' contributions

Authors contributed equally to this article. **AFJ** contributed to data collection, statistical analysis, and drafting the manuscript. **AMJ** contributed to the main idea, design of the study and critical revision.

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