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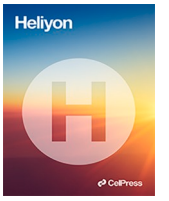

Research article

Emodin prevents renal ischemia-reperfusion injury via suppression of p53-mediated cell apoptosis based on network pharmacology

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ABSTRACT

Background: Previous evidence indicated that emodin has significant advantages for preventing acute kidney injury (AKI). However, the mechanisms responsible for these effects of emodin have yet to be elucidated.

Methods: We first used network pharmacology and molecular docking to identify the core targets of emodin for AKI and performed a range of experiments to validate this result. Pretreatment with emodin for 7 days, the rats were treated with bilateral renal artery clipping for 45 min to identify the prevention effect. Hypoxia/reoxygenation (H/R), and vancomycin - induced renal tubular epithelial cells (HK-2 cells) were treated with emodin to explore the related molecular mechanism.

Results: Network pharmacology and molecular docking showed that anti-apoptosis might be the core mechanism responsible for the action of emodin on AKI; this anti-apoptotic effect appears to because by regulation p53-related signaling pathway. Our data showed that pretreatment with emodin significantly improved renal function and renal tubular injury in renal I/R model rats ($P < 0.05$. The prevention effect of emodin was proved to be related to anti - apoptosis of HK-2 cells, possibly by downregulating the levels of p53, cleaved-caspase-3, pro-caspase-9, and upregulated the levels of Bcl-2. The efficacy and mechanism of emodin on anti - apoptosis was also confirmed in vancomycin - induced HK-2 cells. Meanwhile, the data also showed that emodin promoted angiogenesis in I/R damaged kidneys and H/R-induced HK-2 cells, which was associated with decreasing HIF-1α levels and increasing VEGF levels.

Conclusions: Our findings indicated that the preventive effect of emodin on AKI is probably attributable to anti-apoptosis response and promoting angiogenesis effect.

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1. Background

Acute kidney injury (AKI) continues to represent a severe problem among hospitalized patients and 50% of critically ill patients are known to develop AKI [1]. Although renal replacement therapy has improved the survival rate of patients with AKI, almost 2 million people die from AKI each year. Worryingly, no current recommendations for drugs to prevent AKI in adults, although the high risk of AKI has been recognized [2]. Current therapies are associated with only supportive treatment [3,4], thus highlighting the critical need to develop novel prevention strategies.

Traditional Chinese medicine (TCM) has unique advantages in improving the renal prognosis of AKI due to its characteristics of multiple functions. Emodin is a bioactive compound that can be extracted from *Rheum palmatum*, a traditional form of Chinese medicine, and is known to exert anti-inflammation and antioxidant properties [5–7]. Recent evidence has emerged to indicate the prevention potential of emodin on AKI [8], and the prevention action of emodin on AKI may occur by the regulation of oxidative stress, cell apoptosis and endoplasmic reticulum stress (ERS) [9,10] in human renal tubular cells. However, the mechanisms underlying these processes in renal cells remain very unclear.

Previous research has shown that network pharmacology represents a promising method with which to discover relevant targets and new drugs for specific diseases [11]. In addition, molecular docking represents a significant tool for structural molecular biology and the computer-aided design of new drugs [12]. A previous study showed that emodin exerted a significant influence on the prevention action of AKI [13]. However, the mechanism by which emodin can be used to prevent AKI remains unclear. In the present study, we used a combination of network pharmacology and molecular docking to investigate the possible mechanism of emodin in the prevention of AKI. We focused on relevant targets, pathways and the specific relationship between emodin, apoptosis and angiogenesis. Moreover, we carried out a range of *in vitro and in vivo* experiments to validate these results.

2. Methods

2.1. Target selection

We used the Online Mendelian Inheritance in Man database (OMIN; http://www.omim.org/) and Therapeutic Target Database (TTD, https://db.idrblab.org/ttd/) to acquire AKI-associated target genes and then used the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/) and Zinc (https://zinc.docking.org/) to identify emodin-related target genes. Next, we created a compound-target network with Cytoscape 3.7.1 software (http://www.cytoscape.org/) to identify potential targets. Then, we carried out Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for the enrichment of predicted core targets using the Database for Annotation Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) and the OmicShare tool (http://www.omicshare.com/tools/).

2.2. Component-target molecular docking

The Standard Delay Format (SDF) structure profile of emodin (MOL000472) was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and converted into a Program Data Base (PDB) profile by Open Babel version 2.3.2 software (http://openbabel.org/). The receptor protein for TP53 (PDBID: 3Q05) was then obtained from the Protein Data Bank database (http://www.rcsb.org/pdb). PYMOL version 2.3.4 software (https://pymol.org/2/) was then used to remove water molecules and isolate proteins, and AutoDock Tools version 1.5.6 software (https://www.cgl.ucsf.edu/) was used to add non-polar hydrogen and balance the charges. AutoDock Tools 1.5.6 software was then used to process each receptor protein, adjusting the number of grid points in each direction of the protein, the center of the binding pocket, and the spacing of the grid points. Autodock Vina version 1.1.2 (https://www.cgl.ucsf.edu/) was then used to dock emodin with TP53.

2.3. Animals and experimental design

52 male Sprague Dawley (SD) rats (7–8 weeks, 200 ± 20 g), purchased from Chengdu Dashuo Biotechnology Co., Ltd. This experiment has been approved by the Laboratory Animal Welfare and Ethics Committee. Rats were bred in an animal room with humidity (65–69%), temperature (22–24 °C), and light/dark cycle (12 h). After one week, we randomly divided the rats into sham group (n = 12), model group (n = 20) and emodin group (n = 20). Emodin (≥98%, HPLC grade) was provided by Hubei Xinxin Jiali Biotechnology Co., Ltd. (China), and the emodin suspension (10 mL/kg) was prepared using 0.5% sodium carboxymethylcellulose (CMC-Na). Before the experiment, the plasmatic concentration of emodin was detected by LC-MS/MS, and the results showed that the plasma concentration of emodin ranges from 0.46 ± 0.11 μg ml^{-1} - 3.54 ± 0.87 μg ml^{-1} in 24 h (Fig. S1). The emodin group rats were treated with emodin 30 mg/kg gavage for 7 days according to the bioavailability, metabolism and intervention cycle reported by Liu et al. [14] and Ye et al. [15]. And, the other rats were treated with the same volume of CMC-Na for 7 days. And then, we dissociated the bilateral renal arteries and clipped them for 45 min to stimulate the process of renal ischemia and then restored perfusion for 24 h to stimulate the process of renal reperfusion. And rats in the sham group were treated by only dissociating the bilateral renal arteries without clipping. After reperfusion for 24 h, the rats were sacrificed, aortic blood and kidney were collected.

2.4. Cell culture and experimental groupings

Human renal tubular epithelial (HK-2) cells line (Aiyan Biotechnology Co., Ltd., Shanghai, China) were maintained at 37 °C with CO2 in 45 mL of Dulbecco's modified Eagle medium (DMEM) and the 6th to 9th passages were used for experiments.

We successfully constructed a H/R damaged HK-2 cells and vancomycin-induced damaged HK-2 cells model by sealing for 5 h with tape, and vancomycin treating for 24 h, respectively [16,17]. And then, damaged HK-2 cells were treated with emodin (10, 30, 50 and 80 μM) for 4, 12 and 48 h to investigate its therapeutic effect.

2.5. Histology and immunohistochemistry

Kidneys were fixed in 4% formaldehyde for 24 h, embedded in paraffin, and 4 μm sectioned were cut. Then, the sections were used for histopathological staining of kidney, such as HE and PAS staining. Renal pathological changes were evaluated according to Pallers standard. The scoring criteria are as follows: (1) renal tubular dilatation 1; (2) brush border injury 1 and Loss 2; (3) bullae or cytoplasmic vacuoles 1; (4) interstitial edema 1; (5) exfoliated necrotic cells in the tubular lumen without forming casts or fragments 1 and with forming casts or fragments 2.

The levels of protein in the kidneys were detected by immunohistochemistry (IH). Primary antibodies against VEGF and HIF-1α (Cell Signaling Technology, USA) were prepared in advance. After treatment with 3% H2O2 and goat serum working solution, primary antibody and secondary antibody were added for staining. And then, diaminobenzidine (DBA) (Dako, Denmark)was used was used for color rendering, and hematoxylin was used for counterstaining. Anymicro DSS TM system was used to collect the images.

2.6. Cell vitality assays

Cell Counting Kit-8 (CCK-8), obtained from Dongyuan Chemical Technology Co., Ltd. (Shanghai, China) and used to detected HK-2 cells vitality. HK-2 cells were seeded into 96 well plates and 10 μL of CCK-8 solution was added. The absorbance of the cells at 450 nm was then determined with a microplate reader.

2.7. Cell apoptosis assay

Carboxy fluorescein diacetate, succinimidyl ester (CFSE) and propidium iodide (PI) staining (Sigma, USA) were used to evaluate cell apoptosis. First, HK-2 cells were placed in the wells of a 12-well plate with CFSE and then incubated at 37 °C for 15 min. Next, CFSE (200 μL) was added after removing the CFSE and washing with PBS. Finally, 200 μL of PI was added and detected by fluorescence microscopy (Olympus, Japan).

And TUNEL was used to detected the apoptosis of renal tubular epithelial cells in renal I/R model rats. Kidneys were fixed in 4% formaldehyde for 24 h, embedded in paraffin, and 4 μm sectioned were cut. Then, it was repaired with citric acid for 8 min, washed 2 times with PBS, and incubated with tunel incubation solution (A:B = 1:30) (Roche Group, Switzerland) at 37 °C for 1 h. Added DAPI staining solution for 15 min, mounted with glycerol gelatin, and then stored at −20 °C. Finally, fluorescence microscopy was used to detect.

2.8. Western blotting

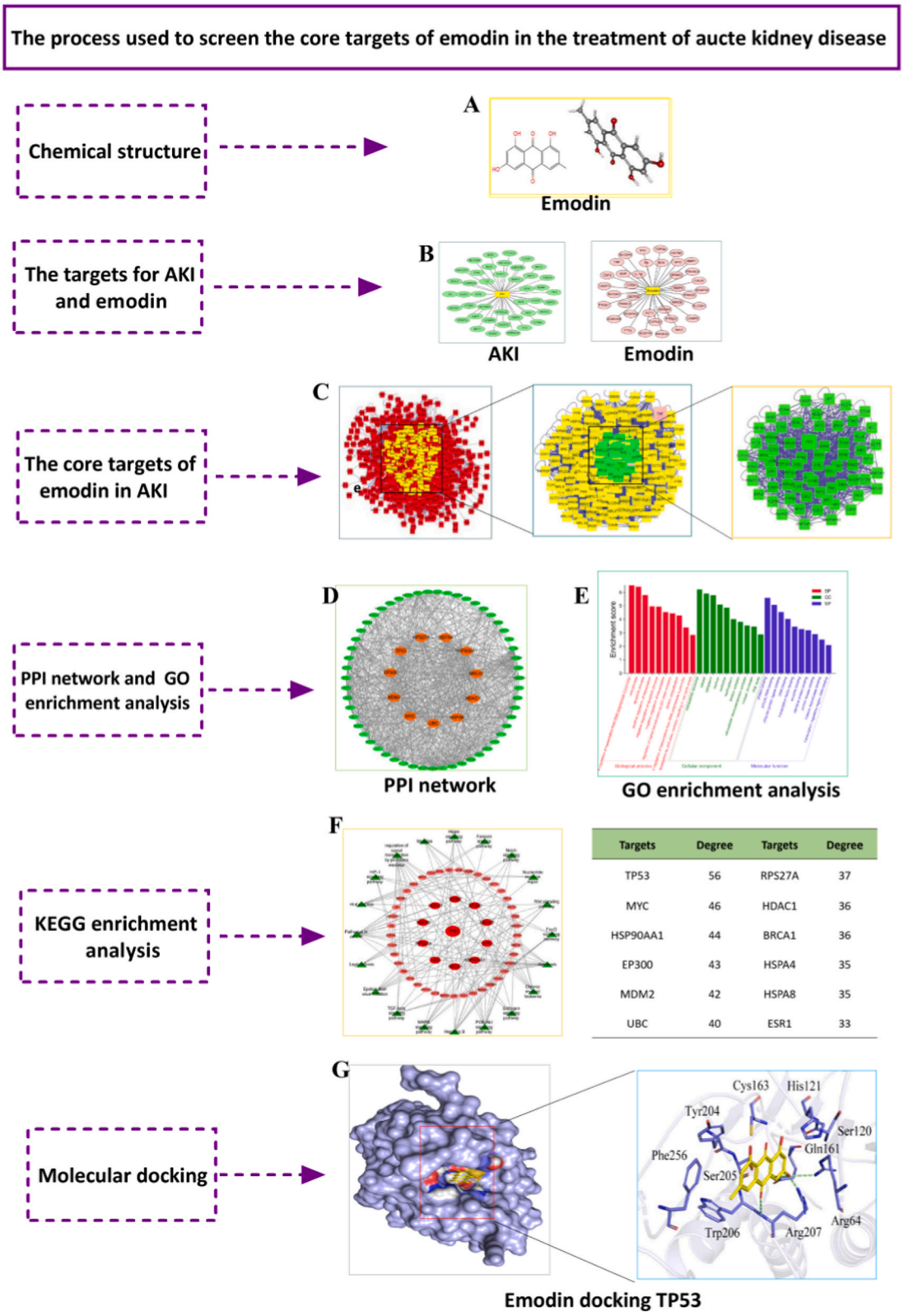
Proteins were extracted by using Cell Total Protein Extraction Kit (Abcam, UK). After separating 20 μL of total protein per well, electrophoresis was performed at 4 °C and 90 V for 1 h–1.5 h. Separated proteins were then migrated to polyvinylidene fluoride (PVDF) membranes and incubated for 1 h with 5% skimmed milk. This was followed by incubation with primary antibodies. After washing 3 times with PBST, a secondary antibody was added to PVDF membranes and incubated with the membranes for 1.0 h at room temperature. The membranes were washed three times with PBST and then were covered with photographic film. Finally, a Gel-Pro Analyzer 4.0 (Media Cybernetics, WDC, USA) was used to quantify protein levels. β-actin was used as a positive control [18].

2.9. Real-time quantitative polymerase chain reaction

Total RNA was obtained using a combination of Trizol (Sangon Biotech, Shanghai, China) and chloroform. Next, 1 mL of the upper phase was mixed with 1 mL of isopropanol and centrifuged at 12,000 g and 4 °C for 5 min. Subsequently, 75% ethanol was used to precipitate the RNA which was subsequently resuspended in 50 μL of nuclease-free water. Antibodies were added to create 9 μL of system solution; this was incubated for 5 min at 65–75 °C to synthesize cDNA. Next, the cDNA was diluted 30 times and stored at −20 °C. Finally, 10 μL of cDNA was added to 96-well plates and placed in a PCR System (ThermoFisher, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control [19].

2.10. Statistical analysis

Statistical Product and Service Solutions software version 20.0 (Software, USA) was applied for statistical analysis. The data were expressed as means ± standard deviation. Normally distributed data were analyze by t-tests for pairwise comparisons. If data were not normally distributed, then we used Wilcoxon's test for pairwise comparisons; $p < 0.05$ was statistically significant.



Targets	Degree	Targets	Degree
TP53	56	RPS27A	37
MYC	46	HDAC1	36
HSP90AA1	44	BRCA1	36
EP300	43	HSPA4	35
MDM2	42	HSPA8	35
UBC	40	ESR1	33

(caption on next page)

Fig. 1. The process used to screen the core targets of emodin in the treatment of aucte kidney disease (AKI). (a): Chemical structure of emodin; (b): The targets for AKI and emodin; (c): The process used to identify the core targets of emodin in AKI; (d): A PPI network showing 66 targets (genes). The orange nodes represent important hub genes. The green nodes represent other genes; e: GO enrichment analysis of 53 nodes, including BPs, MFs and CCs; (f): The target-pathway network of emodin (The outermost triangles represent related pathways; The red nodes represent the important core nodes while the pink nodes represent the other nodes); (g): Emodin docking p53.

3. Results

3.1. The core targets of emodin in the treatment of AKI

Emodin is a signal component extracted from *Rheum palmatum*, a traditional Chinese medicine. The molecular weight of emodin is 270.24 g/mol, and the molecular formula is $C_{15}H_{10}O_5$; the structure of emodin is given in Fig. 1A. 42 AKI-related targets were acquired

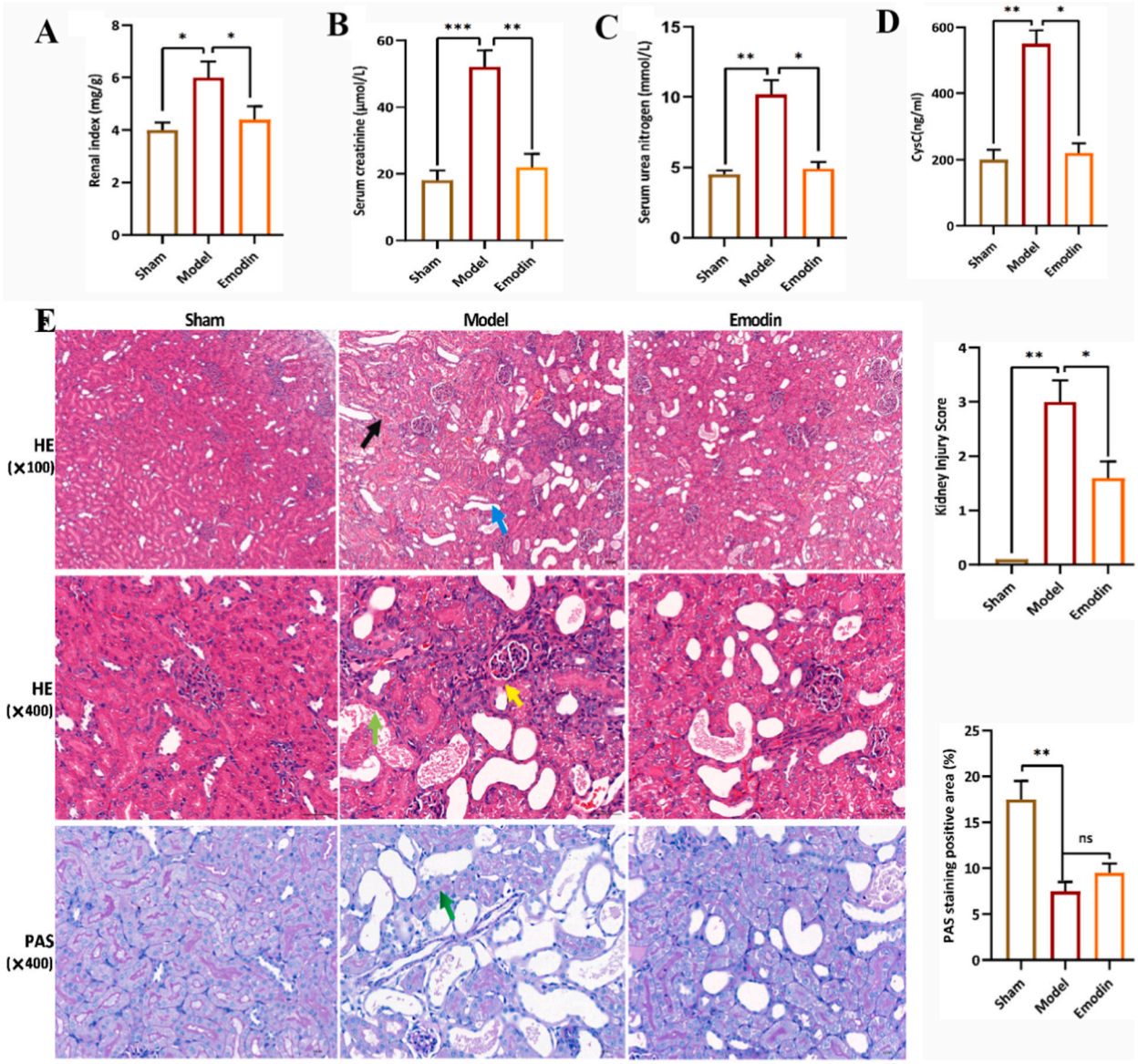


Fig. 2. Emodin improved renal function and alleviated renal injury in renal I/R model rats. **(A–D)** The effect of emodin on the renal index (kidney weight (mg)/rats weight, serum creatinine, urea nitrogen and CysC (Cystatin C) in renal I/R model rats. **(E)** Emodin alleviated pathological damage of the kidney, including renal tubular dilatation (blue arrow), epithelial cell necrosis (black arrow), glomerular basement membrane thickening (yellow arrow), brush cell damage (light green arrow), etc. I/R: ischemia/reperfusion; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. The green arrow represents PAS staining positive area.

from the TTD and OMIM databases, and 37 emodin-related targets were acquired from the Zinc and TCMSP databases (Fig. 1B). And then, the protein-protein interactions (PPI) network was constructed. By considering the 'degree', 'betweenness' and 'closeness centrality', 66 core targets were screened out from a total of 699 targets that could be the core targets of emodin in the treatment of AKI (Fig. 1C). The PPI network indicated that there were interactions between 66 targets; the hub genes were identified as TP53 (Fig. 1D). GO enrichment analysis (Fig. 1E) identified several biological processes (BPs), including the regulation of signal transduction by p53 class mediators, viral processes, and apoptotic processes; cellular components (CCs) mainly included extracellular exosomes, cytosol, and cytoplasm; molecular functions (MFs) was mainly enriched in protein binding, poly (A) RNA binding and enzyme binding. KEGG enrichment analysis (Figure F) demonstrated that the identified targets were mainly involved in pathways related to apoptosis, pathways in cancer and the regulation of signal transduction by TP53 class mediators. Of these, apoptosis was the predominant pathway used by emodin in the treatment of AKI. Molecular docking was used to verify whether emodin inhibited HK-2 cells apoptosis in AKI via regulating p53. Fig. 1G shows that emodin had a high affinity for TP53 and that emodin formed hydrogen bonds with the amino acid residues Leu264, Gly262, Glu258, Arg158, Thr256 and Ser9 in order to stabilize the structure. These findings were further validated by subsequent experiments, as described in Sections 3.2–3.6.

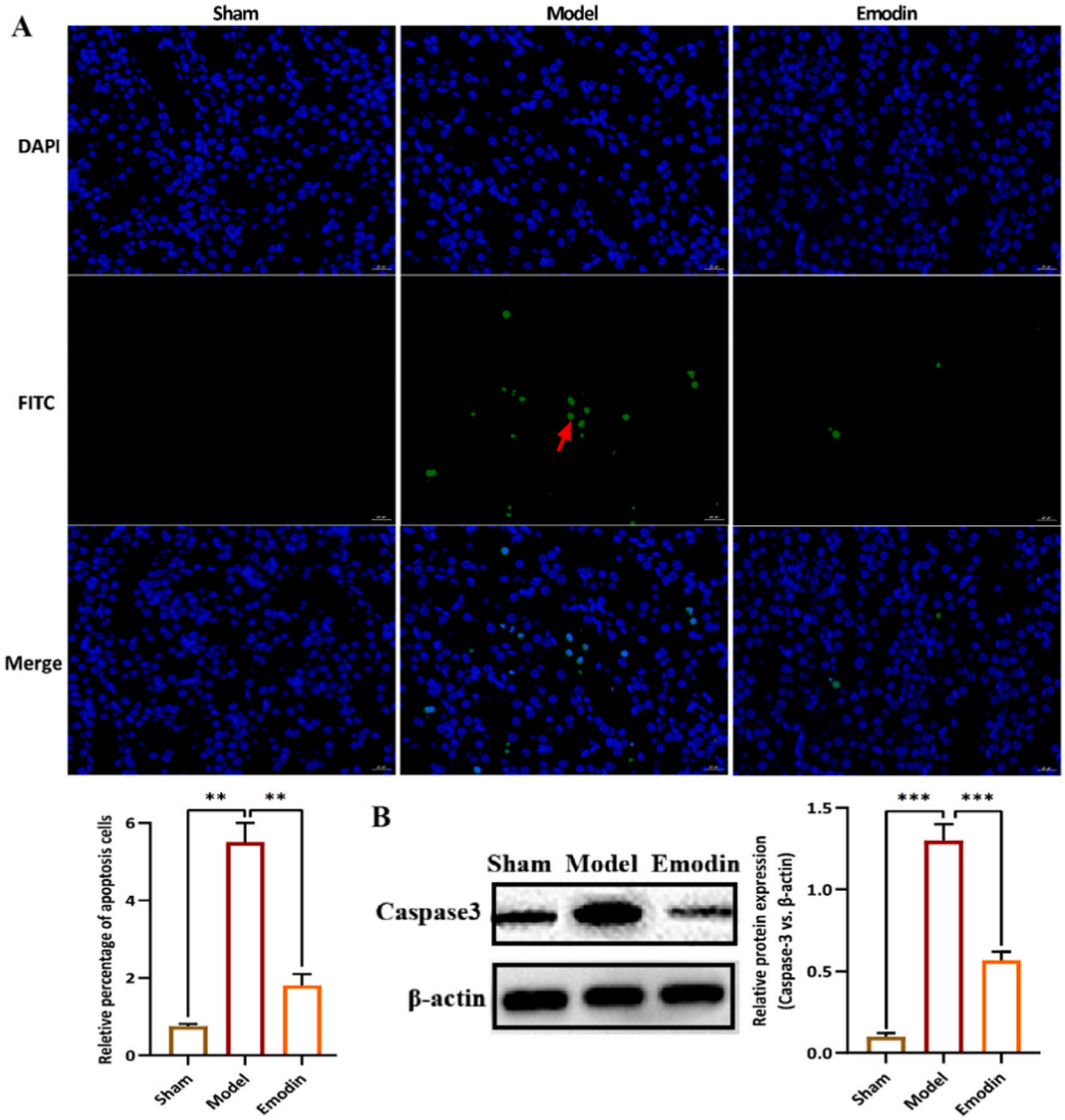


Fig. 3. Emodin inhibited the apoptosis of renal tubular epithelial cells in renal I/R rats. **(A)** TUNEL was used to detect the apoptosis (red arrow) of renal tubular epithelial cells. **(B)** The effect of emodin on apoptosis executing protein Caspase-3 detected by WB. I/R: ischemia/reperfusion; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

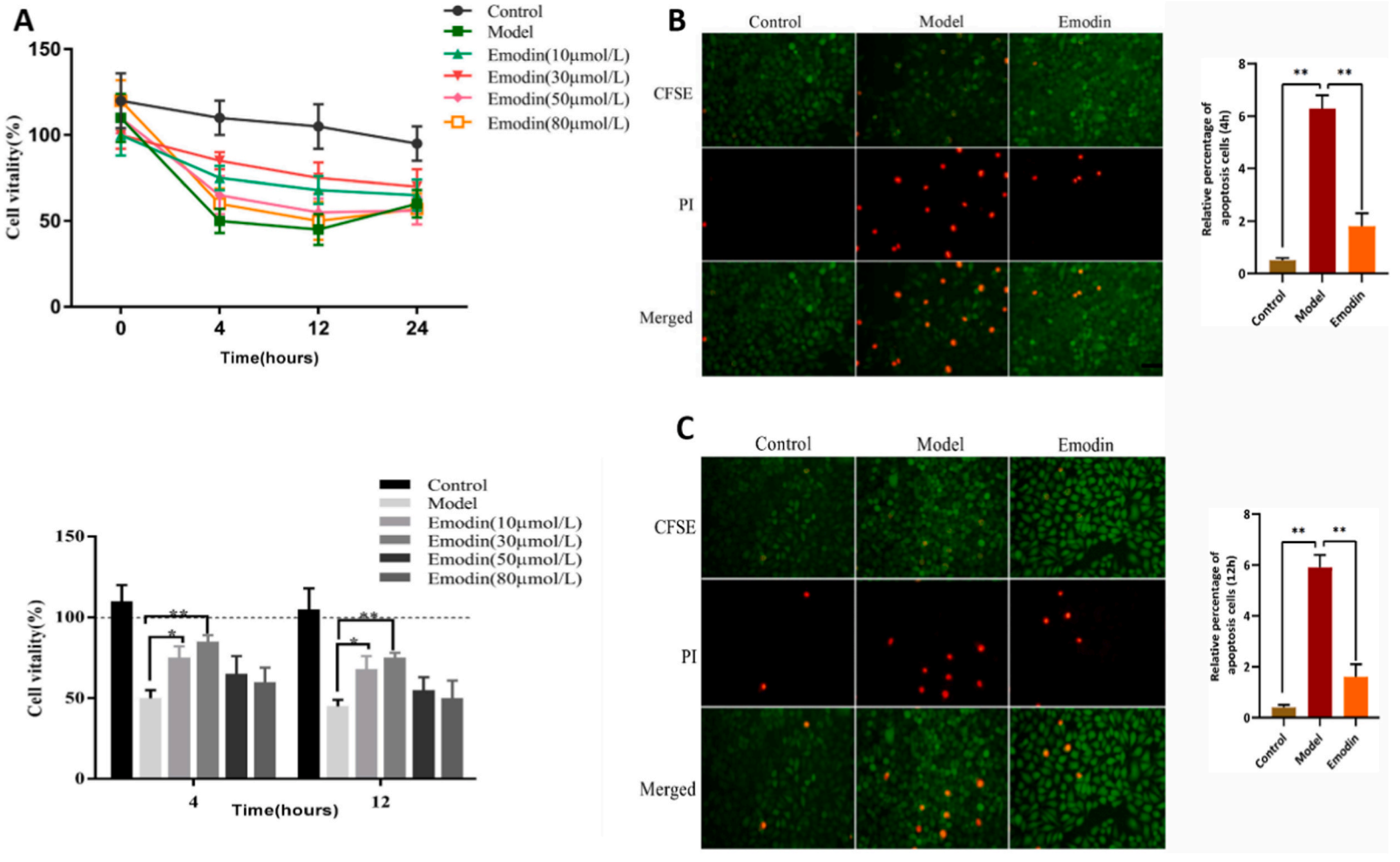


Fig. 4. The effect of emodin on the viability and apoptosis of H/R-induced HK-2 cells. **(A)** The effect of emodin (10, 30, 50, 80 μm) on cell vitality in H/R-induced HK-2 cells. Treatment with 10 and 30 μM emodin significantly inhibited the damage in H/R-induced HK-2 cells. And 30 μM has used this study. **(B)** The effect of emodin (30 μm) on cells apoptosis in H/R-induced HK-2 cells at 4 h. **(C)** The effect of emodin (30 μm) on cells apoptosis in H/R-induced HK-2 cells at 12 h $^{*}P < 0.05$. $^{**}P < 0.01$; H/R, hypoxia/reoxygenation; HK-2, human renal tubular epithelial cell line.

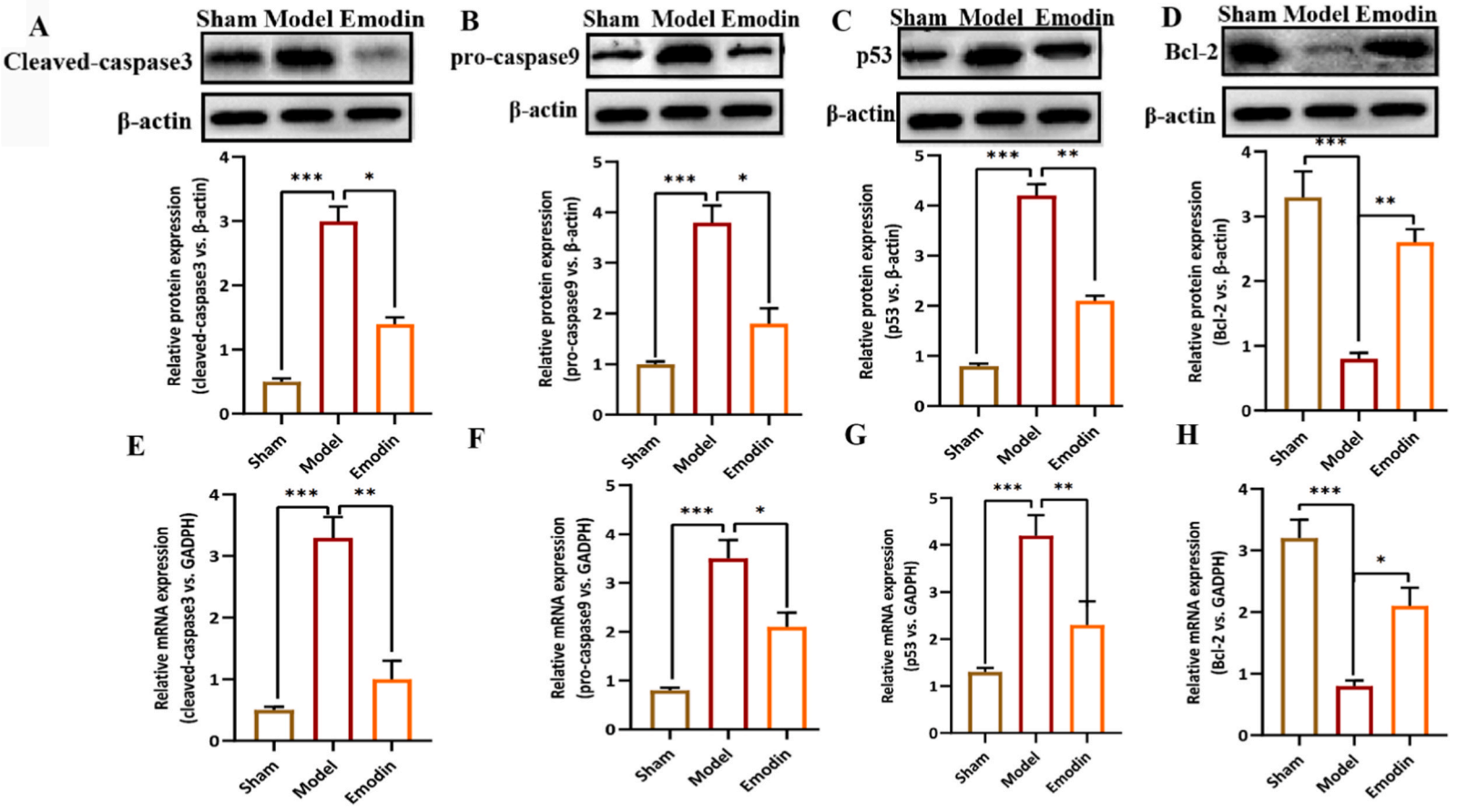


Fig. 5. Emodin regulated the levels of p53, Bcl-2, cleaved-caspase-3/Caspase-3 and pro-caspase-9/caspase-9 in renal I/R model rats. **(A–D)** The effect of emodin on the protein levels of p53, cleaved-caspase-3, pro-caspase-9, and Bcl-2. **(E–H)** The effect of emodin on the *mRNA* levels of *p53, cleaved-caspase-3, pro-caspase-9,* and *Bcl-2*. I/R: ischemia/reperfusion; β-actin and GAPDH were used as internal control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3.2. Emodin improved renal function and renal injury in renal I/R rats

The bilateral renal artery clip for 45 min and reperfusion for 24 h was successfully done in 14 cases (the model group) and 16 cases (the emodin group). As shown in Fig. 2, the renal index (kidney weight (mg)/body weight (g)) was obviously higher in the model group than that in the sham group, and pretreatment with emodin decreased the renal index (Fig. 2A). And the rats in the model group developed significant serum urea nitrogen, serum creatinine and Cystatin C, which was obviously reduced after pretreating with emodin for 7 days (Fig. 2B, C, D).

We further analyzed the changes in kidney pathology, and the results showed that The renal I/R rats developed obvious renal injury, including renal tubular dilatation, epithelial cell necrosis, glomerular basement membrane thickening and brush cell damage, which was attenuated by pretreatment with emodin for 7 days (Fig. 2E). This finding indicated emodin alleviated renal function and renal injury in renal I/R rats.

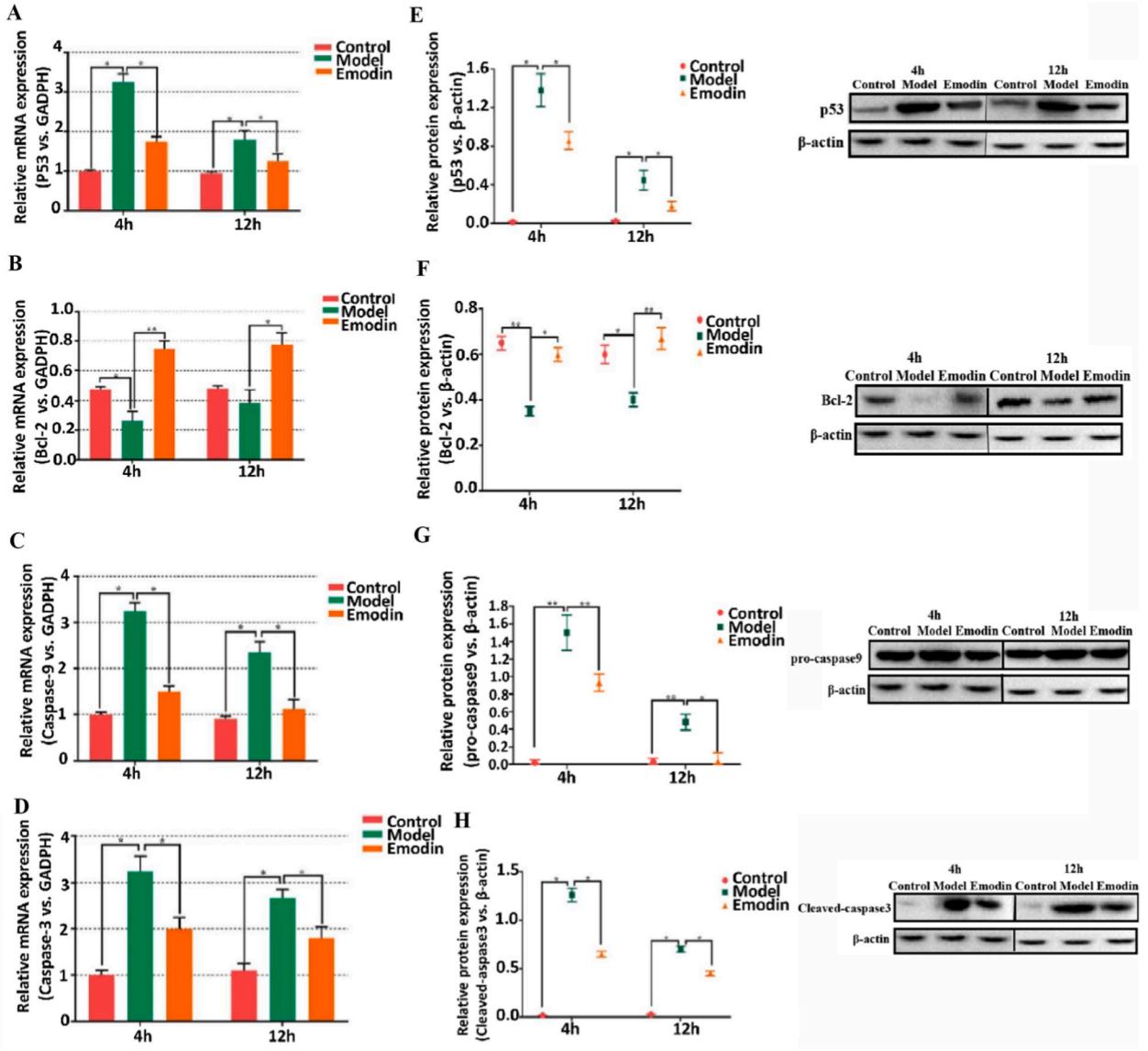


Fig. 6. Emodin regulated the levels of p53, Bcl-2, cleaved-caspase-3/Caspase-3 and pro-caspase-9/caspase-9 in H/R-induced HK-2 cells. **(A–D)** The effect of emodin (30 μm) on the *mRNA* levels of *p53, cleaved-caspase-3, pro-caspase-9,* and *Bcl-2*. **(E–H)** The effect of emodin (30 μm) on the protein levels of p53, cleaved-caspase-3, pro-caspase-9, and Bcl-2. H/R: hypoxia/reoxygenation; HK-2, human renal tubular epithelial cell line; GADPH and β-actin were used as internal control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

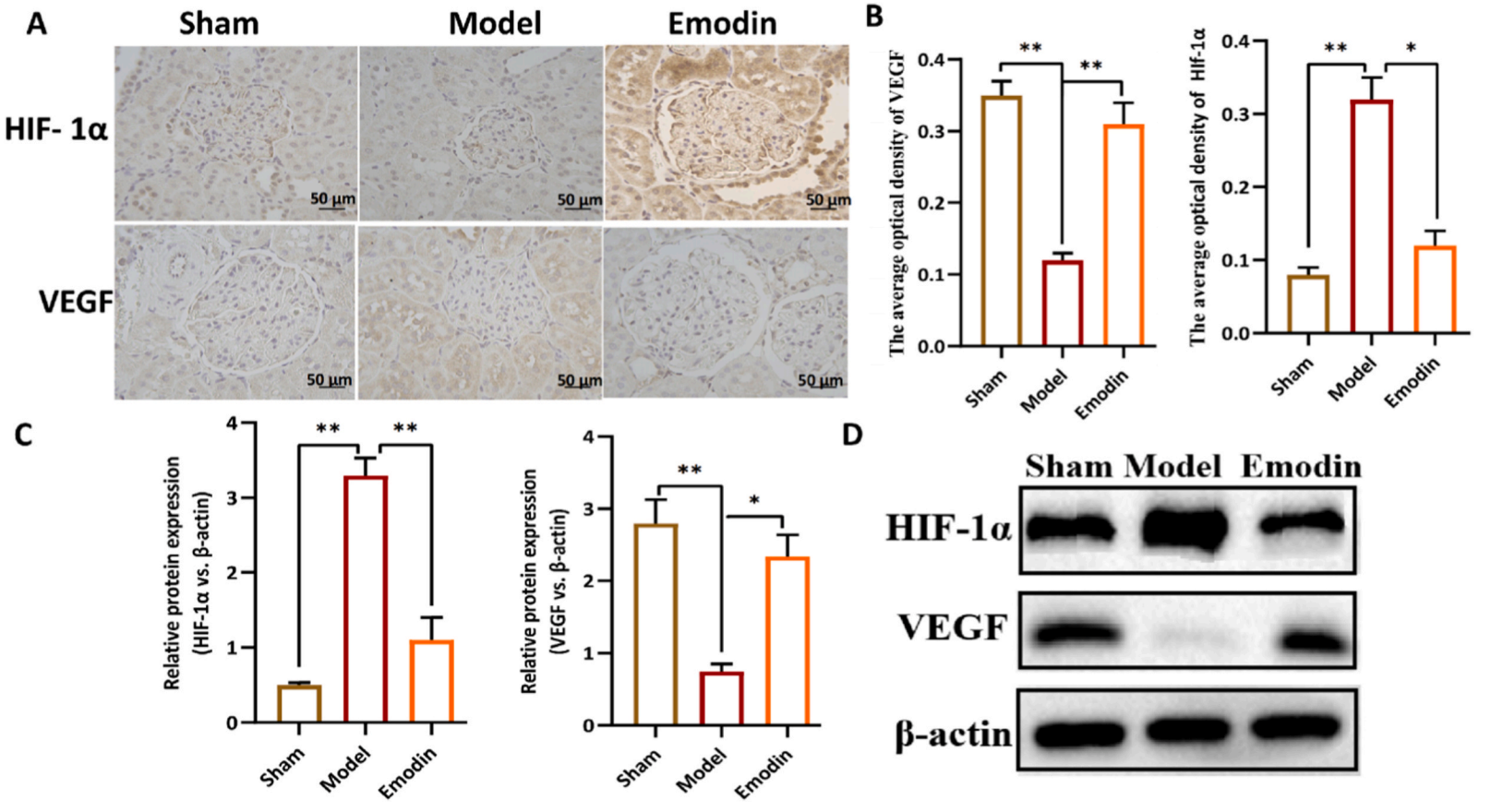


Fig. 7. Emodin regulated the levels of HIF-1α and VEGF. **(A–D)** The effect of emodin on the protein levels of HIF-1α and VEGF in renal I/R model rats. I/R: ischemia/reperfusion; β-actin were used as internal control. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

3.3. Emodin inhibited the apoptosis of renal tubular epithelial cells in renal I/R rats

In order to explore whether emodin can improve renal injury in renal I/R rats by inhibiting apoptosis, we detected apoptosis of renal tubular epithelial cells. Notably, it showed that apoptotic chromatin condensation was obviously observed in the model group, indicating that renal tubular epithelial cells developed significant apoptosis after exposure to I/R (Fig. 3A). Pretreatment with emodin inhibited the apoptosis of renal tubular epithelial cells induced by I/R. Meanwhile, the apoptosis executing protein, Caspase-3, was increased in renal I/R rats, which experienced an obvious decrease after pretreating with emodin (Fig. 3B). The finding showed that the role of emodin in improving renal injury was closely related to its anti-apoptotic effect.

3.4. Emodin inhibited apoptosis in HK-2 cells following H/R injury

The cell viability of HK-2 cells showed obvious reduction following H/R ($P < 0.05$) (Fig. 4A); treatment with 10 and 30 μM emodin significantly inhibited the damage (all $P < 0.05$) mediated by H/R at 4 h and 12 h; the 30 μM emodin treatment presented with the most significant efficacy (Fig. 4A, $P < 0.01$). However, the effects were weaker at concentrations of 50 and 80 μM, indicating that pretreatment with ≧ 50 μM of emodin did not markedly improve the viability of HK-2 cells when damaged by H/R. Therefore, 30 μM of emodin was used for all subsequent experiments.

Next, we investigated whether emodin alleviated injury in HK-2 cells mediated by H/R by inhibiting the apoptotic process. To do this, we used CFSE/PI to monitor the apoptosis of HK-2 cells, and the data showed that HK-2 cells experienced significant apoptosis following H/R injury, while treatment with emodin (30 μM) visibly inhibited H/R-induced apoptosis at 4 h and 12 h, shown in Fig. 4B and C. These findings indicated that the protective effect of emodin on HK-2 cells was associated with anti-apoptosis.

3.5. Emodin regulated the levels of p53, cleaved-caspase-3/Caspase3, pro-caspase-9/caspase-9 and Bcl-2 in renal I/R rats

Apoptosis may be the key mechanism of emodin in the treatment of AKI, and p53 may be the main target of emodin in the inhibition of I/R-induced apoptosis in renal tubular epithelial cells. As demonstrated in Fig. 5A and E, the protein and *mRNA* levels of p53 in the kidney were evidently increased after renal I/R ($P < 0.05$); pretreatment with emodin led to a significant downregulation of p53 protein expression ($P < 0.05$). These findings indicated that emodin inhibited renal I/R-mediated renal tubular epithelial cells apoptosis probably by regulating p53, at least in part.

As we all know, p53 mediated cell apoptosis by activation of caspase-9 and caspase-3 [20]. Pro-caspase9 and cleaved-caspase3 protein levels were significantly increased in the kidney after exposure to I/R (Fig. 5B and C); the *mRNA* expression of Caspase-3 and Caspase-9 were also increased (Fig. 5F and G); pretreatment with emodin decreased the protein and *mRNA* expressions of pro-caspase9 and cleaved-caspase3; this was also accompanied by an obvious reduction in the levels of p53 (all $P < 0.05$). These findings showed that emodin exerted an anti-apoptotic effect, probably via decreasing p53 protein expression to inhibit the activation of caspase-9 and caspase-3.

Moreover, p53 initiation cell apoptosis is related to regulating the levels of Bcl-2 [21]. As expected, Bcl-2 protein and *mRNA* expression were notably reduced in the renal I/R rats ($P < 0.05$), which experienced a significant increase after pretreatment with emodin for 7 days ($P < 0.05$) (Fig. 5D and H). The upregulated Bcl-2 was accompanied by downregulated p53. These findings showed that emodin inhibited I/R-mediated apoptosis in renal I/R rats probably by downregulating the levels of p53 to increase Bcl-2 levels.

3.6. Emodin regulated the expression of p53, cleaved-caspase-3/Caspase3, pro-caspase-9/caspase-9 and Bcl-2 in H/R-induced HK-2 cells

HK-2 cellls were treated with H/R, and the results showed the protein and *mRNA* levels of p53 was both upregulated at 4 h and 12 h (Fig. 6A and E); the protein and *mRNA* expression of Bcl-2 were downregulated (Fig. 6B and F); the protein levels of pro-caspase9 and cleaved-caspase3 were increased (Figure G and H), and the *mRNA* expression of Caspase-3 and Caspase-9 was also upregulated (Fig. 6C and D). After pretreating with emodin (30 μM) treated, the levels of p53 were decreased in H/R-induced HK-2 cells, which was accompanied by a reduction in cleaved-caspase-3/Caspase-3, cleaved-caspase-9/Caspase-9. Meanwhile, treating with emodin (30 μm) obviously led to a significant upregulation of Bcl-2. The results are consistent with those *in vivo*. These findings showed that emodin inhibited H/R-induced HK-2 cells apoptosis probably via downregulating the levels of p53 to reduce cleaved-caspase-3/Caspase-3, cleaved-caspase-9/Caspase-9 levels, and increase Bcl-2 levels.

3.7. Emodin regulated the levels of HIF-1α and VEGF in I/R damaged kidneys

The hypoxia-inducible factor-1 alpha (HIF-1α) is an oxygen-sensitive transcriptional activator in response to hypoxia, and over-expressed HIF-1α reduce release of vascular endothelial growth factor (VEGF) and its related vascular repair factors to inhibit angiogenesis and cell repire in renal I/R [22,23]. In this study, we detected the levels of HIF-1α and VEGF using IH and WB (Fig. 7A–D), and the results showed that HIF-1α expression was increased and the levels of VEGF was decreased in kidneys after exposure to ischemia, and emodin downregulated HIF-1α levels, and upregulated VEGF levels.

3.8. Emodin inhibited the apoptosis of vancomycin-induced HK-2 cells

In order to further clarify the anti-apoptotic effect of emodin in AKI, we performed experiments in vancomycin-induced HK-2 cells.

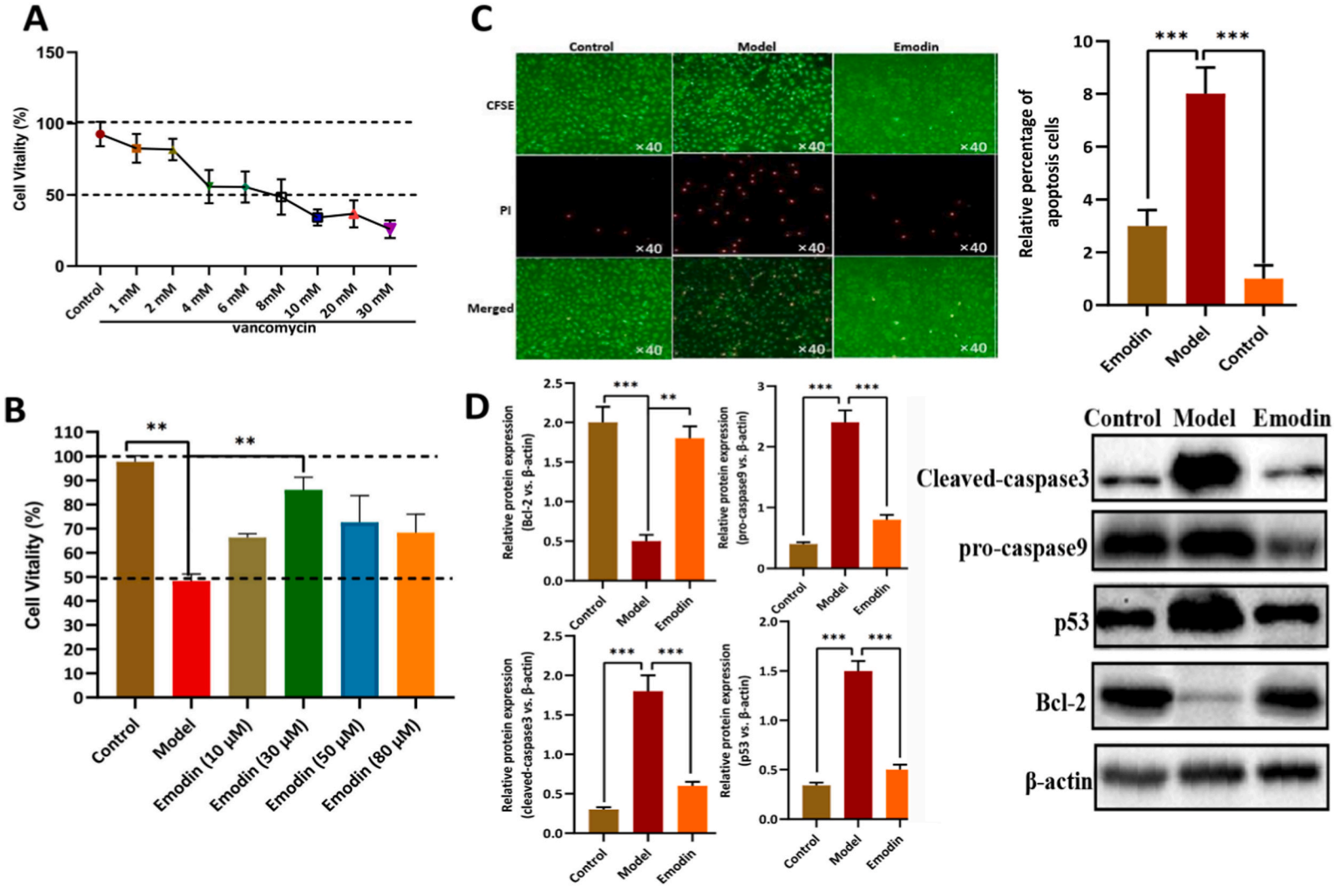


Fig. 8. Emodin inhibited vancomycin - induced HK-2 cells apoptosis via decreasing the levels of p53, cleaved-caspse3, pro-caspase-9, and increasing Bcl-2 levels detected by WB. **(A)** Vancomycin (4 mM) was used to treat HK-2 cells due to CCK-2 results. **(B)** The effect of emodin (10 μM, 30 μM, 50 μM, 80 μM) on cell vitality in vancomycin - induced HK-2 cells. **(C)** The effect of emodin on cell apoptosis induced by vancomycin in HK-2 cells. **(D)** Emodin regulated the levels of p53, cleaved-caspse3, pro-caspase-9, and Bcl-2. HK-2, human renal tubular epithelial cell line; β-actin were used as internal control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

As shown in Fig. 8A, vancomycin (4 mM) was used to construct HK-2 cells damage model according to the results of CCK-8. Subsequently, emodin (10, 30, 50 and 80 μM) treated vancomycin-induced HK-2 cells to investigate anti-apoptotic effect of emodin. As shown in Fig. 8B and C, emodin (30 μM) significantly improved cell viability and cell apoptosis. As shown in Fig. 8D, the levels of apoptosisi - related protein cleaved - caspase3, p53, pro-caspase9 were obviously increased, and Bcl-2 was decreased after treating with vancomycin for 24 h, which experienced a significant improvement after treating with emodin. This findings showed emodin inhibited the apoptosis of vancomycin-induced HK-2 cells via decreasing p53, cleaved-caspase3, pro-caspase9 levels, and increased Bcl-2 levels.

4. Discussion

Prevention strategies of AKI is limited, and there was no current recommendations for drugs to prevent AKI. In this study, emodin pretreatment for 7 days to observe the preventive effect of emodin on AKI. Our data provided evidence that pretreatment with emodin significantly reduced the urinary albumin, serum creatinine, and serum cystatin C, and alleviated the renal tubular injury in a rat model with renal I/R. The prevention effect of emodin on AKI was associated with anti-apoptosis and promoting angiogenesis.

In this study, after pretreating with emodin for 7 days, the rats was treated with bilateral renal artery clipping for 45 min to construct a renal I/R injury model. Compared to the age and gender-matched normal rats, the rats in the model group significantly developed abnormal renal function and renal tubular injury. While, pretreatment with emodin obviously improved renal function and renal tubular injury induced by I/R, indicating the preventive effect of emodin on AKI. Emodin also has advantages in treatment of AKI. Emodin has been reported to improve acute kidney damage caused by various reasons, including I/R, sepsis-associated, and cisplatin-induced et al., which is related to its anti-apoptosis, oxidative stress, endoplasmic reticulum stress, and inflammation [24–26]. However, the molecular mechanism remains unclear.

In this present study, we used network pharmacology to predict the underlying mechanism of emodin for AKI, and the data showed that apoptosis was the predominant pathway used by emodin for AKI. Apoptosis in renal tubular epithelial cells mediated by renal I/R is regarded as a major pathogenic process associated with AKI [27,28]. Preventing apoptosis in HK-2 cells is considered an effective method for treating and preventing AKI [29]. In our study, renal tubular epithelial cells experienced a significant apoptosis in renal I/R rats, which was obviously inhibited after pretreatment with emodin. Meanwhile, emodin (30 μm) was found to exert an obvious anti-apoptosis effect on H/R-induced and vancomycin-induced HK-2 cells, indicating that anti-apoptosis may be the core mechanism of emodin in the prevention of AKI. Emodin can exert anti-apoptotic, anti-inflammatory, and anti-tumor effects [30,31]. In previous research, emodin was proven to inhibit H/R-induced apoptosis in HK-2 cells [32]. Our findings showed that the prevention effect of emodin on renal I/R injury was closely related to anti-apoptosis, which is consistent with previous research [33]. Meanwhile, the anti-apoptotic effect was closely with concentration of emodin due to its nephrotoxicity of emodin on HK-2 cells, which is consistent with previous study [34,35].

Network pharmacology showed that TP53 was the strongest core target of emodin for AKI. It has been confirmed that a p53 knockout mouse (p53-KO) was shown to be resistant to vancomycin-induced AKI [36], and p53 mediates vancomycin-induced AKI by activating miR- 192–5p, an important potential diagnostic marker for AKI [37]. As we all know, apoptosis can be mediated by p53 by mechanisms that are dependent or independent of the activation of p53 [38]. In our experiment, the levels of p53 were increased both in renal I/R model rats, H/R-induced HK-2 cells, and vancomycin-induced HK-2 cells. Emodin was found to exert an obvious anti-apoptotic effect via decreasing p53. Bcl-2, the main anti-apoptotic protein that acts against apoptosis, is inhibited by p53 [39]. The accumulation of p53 serves to repress the expression of Bcl-2, thus leading to reduced anti-apoptotic ability [40]. Our findings showed that after pretreating with emodin, the significant reduction in p53 expression was accompanied by a significant upregulation in Bcl-2 levels, thus confirming that the important role of p53 in the emodin-induced inhibition of apoptosis of HK-2 cells was closely related to the upregulation of Bcl-2. In addition, emodin has been proven to reduce mitochondrial membrane potential, a process that has also been correlated with changes in the expression and localization of the Bcl-2 family of proteins [41,42]. Bcl-2 antagonizes the pro-apoptotic effects of Bax by forming homodimers [43,44]. Our data showed that emodin treatment upregulated the levels of the anti-apoptotic protein Bcl-2, which could be induced by reduced levels of p53. It is also possible that emodin may promote a Bcl-2/Bax imbalance to Bcl-2 [45].

Meanwhile, Caspase-3, a pivotal regulator of apoptosis, can be activated by p53-dependent signaling pathways [46]. Emodin pretreatment reduced the levels of cleaved-caspase-3, pro-caspase-9 and p53 in renal I/R injury model, H/R-induced and vancomycin-mediated HK-2 cells. Caspase-9 is a member of the caspase family; the activation of caspase-9 activates procaspase-3, thus leading to the accumulation of caspase-3. The regulatory effect of p53 on the caspase family has been reported previously, including caspase-9 and caspase-8; the activation of caspase-9 subsequently activated the executive apoptosis protein caspase-3, thus leading to targeted cell death [47,48]. In the present research, our results demonstrated that the expression levels of caspase-9 and p53 were both down-regulated following pretreatment with emodin. Furthermore, p53 was predicted as the core target of emodin for AKI, according to network pharmacology and molecular docking, thus proving that the down-regulation of p53 induced by emodin in renal I/R models, H/R-damaged and vancomycin-mediated HK-2 cells caused the suppression of caspase-9 and then inhibited the activation of caspase-3. It was also found that the suppression of caspase-9 repressed the lysis of downstream pro-caspase, thus resulting in the inhibition of caspase-3.

HIF-1α is an oxygen-sensitive transcriptional activator in response to hypoxia [49]. HIF-1α/VEGF signaling pathway has been previously confirmed to play a critical role in both angiogenesis and various diseases, including AKI [50,51]. The role of HIF-1α/VEGF axis in angiogenesis has been recognized, and the role in the renal tissues injury has been confirmed, where it promotes angiogenesis [52]. In this study, we found the abnormal expression of HIF-1α and VEGF in renal IRI and H/R-induced HK-2 cells, indicating an less

active angiogenesis. Pretreatment with emodin decreased HIF-1α levels and increased VEGF levels to promote angiogenesis. This findings showed that emodin improved HK-2 cells injury probably via regulating HIF-1α/VEGF pathway to promote angiogenesis. In addition, the level of Bcl-2 is regulated by HIF-1α, which is contributed to reducing hypoxia - mediated apoptosis. The preventing effect of emodin on I/R damaged HK-2 cells was closely associated with promoting angiogenesis effect.

There are some limitations in this present study. Although the findings showed that p53 were a key target of emodin in the treatment of AKI via network pharmacology and experiment verification, it needs further experiments to confirm that emodin prevents apoptosis of I/R-induced HK-2 cells via targeting p53, such as gene intervention. Meanwhile, p53 is a multifunctional protein, which participates in the process, including cell cycle, cell apoptosis, and DNA damage repair et al. However, we only focused on cell apoptosis, which may lead to one-sided research results. Moreover, we focused on the role of emodin in a H/R-induced and vancomycin-induced apoptosis model in HK-2 cells, there are still some deficiencies in the experiment section for limiting animal models. In the next experiment, we will focus on various types of AKI models.

5. Conclusion

We used network pharmacology, molecular docking, *in vitro and vivo* experiments to evaluate the prevention efficacy and mechanistic of emodin on AKI, which is probably attributable to anti-apoptosis response and promoting angiogenesis effect via regulating p53/Caspase-9/Caspase-3, p53/Bcl-2 and HIF-1α/VEGF signaling pathway (Fig. 9).

Declarations

Author contribution statement

Hongmei Lu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Yuhua He: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Dengpiao Xie: Performed the experiments; Wrote the paper.
Bo Qu: Performed the experiments.
Mingquan Li: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supp. material/referenced in article.

Consent for publication

All authors approved the publication of this manuscript.

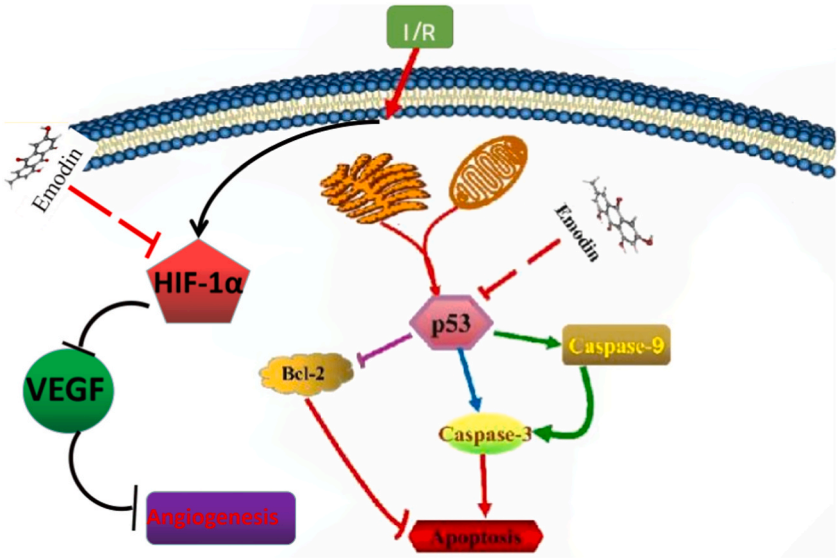


Fig. 9. The prevention of emodin on AKI is probably attributable to anti-apoptosis response and promoting angiogenesis effect. Emodin inhibited I/R-mediated apoptosis, via regulating p53 - related signaling pathway, including p53/bcl-2 signaling pathway (the purple), p53/caspase-3 signaling pathway (the blue), and p53/caspase-9/caspase-3 signaling pathway (the green). Emodin promote angiogenesis via HIF-1α/EVGF aix (the black). I/R: ischemia/reperfusion.

Availability of data and materials

The datasets used during the present study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that there are no conflicts of interest.

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Supplementary materials

The targets of emodin in the treatment AKI are shown in Table S1. GO enrichment analyses are shown in Table S2 while KEGG enrichment analyses are shown in Table S3. The results of Western blot was shown in Figs. S1–23.

Acknowledgments

Mingqua Li and Weijing Liu designed and supervised the study. Hongmei Lu, Dengpiao Xie and Bo Qu performed the experimental work. Hongmei lu and Yuhua He performed the analysis. Hongmei Lu and Dengpiao Xie drafted the manuscript. Hongmei Lu and Yuhua He contributed equally to this work.

Abbreviations

AKI	Acute kidney injury
H/R	Hypoxia/reoxygenation
HK-2 cells	Renal tubular epithelial cells
Bcl-2	B-cell lymphoma-2; p53: p53 tumor suppressor protein
Caspase-3	Cysteine aspartic acid-specific protease 3
Caspase-9	Cysteine aspartic acid-specific protease 3
HIF-1α	Hypoxia-inducible factor-1 alpha
VEGF	vascular endothelial growth factor
RIRI	Renal ischemia reperfusion injury
I/R	ischemia reperfusion
OMIN	Online Mendelian Inheritance in Man
TTD	Therapeutic Target Database
TCMSP	Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform
GO	Gene Ontology
KEGG	Kyoto Encyclopedia ofGenes and Genomes
CCK-8	Cell Counting Kit-8
BPs	Biological processes
CCs	cellular components
MFs	Molecular function
PPI	protein–protein interactions
RT-qPCR	Real-time quantitative polymerase chain reaction
DAVID	Database for Annotation Visualization and Integrated Discovery

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15682.

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