Molecular docking and *in vivo* studies of liquiritin against acute myocardial infarction via TLR4/MyD88/NF-κB signaling

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SHORT COMMUNICATION

Abstract

Licorice (*Glycyrrhiza glabra L.*) is an essential herb in Chinese medicine, as well as a common ingredient in health foods and natural sweeteners. Liquiritin, the primary constituent of licorice, possesses a wide range of pharmacological and biological properties. This research aims to study the protective mechanism of liquiritin in the myocardium. The potential therapeutic efficacy of liquiritin against acute myocardial infarction (AMI) was tested using molecular docking and verified using an AMI rat model caused by the ligation of the LAD coronary artery. Molecular docking between liquiritin and toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88) was predicted using SystemsDock. Then, for experimental validation, *in vivo* studies were employed. Rats with the AMI model established by ligation of left anterior descending coronary artery were divided into four groups—sham group, model group, captopril group, and liquiritin group. LVSP, LVEDP, +dp/dtmax, and -dp/dtmax were detected and analyzed. HE and Masson staining were used to observe the pathological changes. The protein expressions of TLR4, MyD88, and nuclear factorκB p65 (NF-κB p65) were detected by Western blotting. Molecular docking showed that liquiritin may act on the TLR4 and MyD88, and, therefore, liquiritin was predicted to exert anti-inflammatory effects by regulating the TLR4/MyD88 signaling pathway. Liquiritin improved LVSP, +dp/dtmax, -dp/dtmax, and LVEDP levels, and alleviated pathological changes and cardiac fibrosis. Further study found that liquiritin could decrease the overexpression of TLR4, MyD88, and NF-κB, which validated the molecular docking study. Hence, liquiritin ameliorates AMI by reducing inflammation, and blocking TLR4/MyD88/NF-κB signaling. These results indicate that liquiritin as a potential compound could alleviate AMI and broaden its application.

Keywords: acute myocardial infarction; liquiritin; molecular docking; TLR4/MyD88/NF-κB signaling

Introduction

Acute myocardial infarction (AMI) is a major manifestation of ischemic heart disease (IHD), which is a leading cause of chronic heart failure (CHF) (Lin *et al.*, 2020). As a serious cardiovascular event, AMI has become the leading cause of death in the world, which is characterized by high morbidity and mortality. Therefore, it needs to

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be given enough attention, and it is particularly important to find an appropriate treatment (Yousufuddin *et al.*, 2019). The occurrence of AMI is related to many factors, such as arrhythmia, severe inflammatory response, and cardiac dysfunction (Sinnecker *et al.*, 2016). At present, the surgical treatment of AMI mainly includes reperfusion and revascularization therapy (Horikoshi *et al.*, 2021). Restoring coronary blood flow to the infracted myocardium can significantly reduce myocardial infarction; however, this process may further cause myocardial ischemia/reperfusion (I/R) injury, which causes secondary damage to AMI patients (Davidson *et al.*, 2019; Yang *et al.*, 2019). In spite of modern drugs for the prevention and treatment of AMI and improved public awareness, there is still a need for new and safe drugs to prevent AMI. Therefore, it is particularly important to find and develop new cardioprotective Traditional Chinese Medicine (TCM) for AMI patients.

Epidemiological evidence has suggested that diets rich in fruits and vegetables are associated with a lower incidence of cardiovascular diseases, as fruits and vegetables are rich in flavonoids and flavonoid glycosides (Krga *et al.*, 2016; Zhou *et al.*, 2020). Recent studies have found a positive correlation between higher intakes of flavonoids and reduced cardiovascular disease mortality (Yamagata, 2019). Licorice (*Glycyrrhiza glabra* L.) is an essential herb in Chinese medicine, which is also widely used in health foods and natural sweeteners (Jiang *et al.*, 2020; Kwon *et al.*, 2019). Licorice has anti-inflammatory, anti-obesity, anti-oxidant, anti-viral, and neuroprotective properties (Ahmed-Farid *et al.*, 2019; Ojha *et al.*, 2015; Sun *et al.*, 2019). Flavonoids and flavonoid glycosides are one of the main chemical components of licorice. Liquiritin, as a flavonoid glycoside, is also one of the main components of licorice, which possesses anti-myocardial fibrosis, anti-cancer, anti-oxidative and neuroprotective effects (Huang *et al.*, 2018; Sun *et al.*, 2010; Wei *et al.*, 2017). Previous studies have reported that liquiritin could suppress the levels of type I collagen, type II collagen, and alpha-smooth muscle-actin (α-SMA), reduce the release of inflammatory cytokines such as TNF-α, IL-6, and IL-17, and inhibit the protein expression of nuclear factorκB (NF-κB) phosphorylation via regulating IKKα/IκBα signaling pathway. It also has a protective effect against myocardial fibrosis (Zhang *et al.*, 2016). Our previous studies found that liquiritin could directly inhibit ATE1 overexpression and inhibit TAK1 and JNK1/2 phosphorylation in H9c2 transfected by pcDNA3.1/ATE1, which plays a role in reducing Ang II-induced cardiomyocyte hypertrophy due to its regulation of ATE1/ TAK1-JNk1/2 pathway (Mo *et al.*, 2022).

Toll-like receptor 4 (TLR4) can activate the expression of pro-inflammatory factors and chemokines by regulating the MyD88-dependent pathway, which affects many diseases, including cardiovascular diseases, allergic diseases, neuronal degeneration, and autoimmune diseases (He *et al.*, 2019; Mian *et al.*, 2019). Especially, in AMI, the pathogenesis is that the inflammatory response caused by activated TLR4 may be because of the TLR4-Myd88-dependent signaling pathway. Molecular docking can predict the potential target of the natural products, and then verify it through experiments, which can explain the mechanism of action of the natural products from Chinese Medicine.

Therefore, in this study, molecular docking technology was used to find whether liquiritin is a potential inhibitor of TLR4 and Myd88. The purpose of this study was to screen liquiritin for potential therapeutic targets for TLR4 and Myd88 through molecular docking, then determine possible pathological pathways, find the mechanism of interaction between liquiritin and receptors, and confirm results by an *in vivo* assay.

Materials and Methods

Animals

Sprague-Dawley (SD) rats (Male, weight 200±20 g, SCXK 2017-001) were purchased from the Experimental Animal Center of Anhui Medical University. All rats were housed under 23±2°C, 12/12 h light/dark cycles. All experimental procedures were followed by the Center of Scientific Research of Anhui University of Chinese Medicine.

Chemicals

Liquiritin was purchased from Shanghai Yuanye Biotechnology Company (Shanghai, China), and the purity was greater than 98%. TLR4 was from Affinity Bioreagents (Golden, CO). MyD88 and NF-κB p65 were from Abbkine (China).

Molecular docking

The liquiritin structure was obtained from the PubChem website. TLR4 (PDB ID: 3VQ2) (Ohto *et al.*, 2012) and MyD88 (PDB ID: 4DOM) (Snyder *et al.*, 2013) structures were obtained from RCSB (www.rcsb.org/pdb). SystemsDock was used for molecular docking (Hsin *et al.*, 2016). The method of molecular docking consisted of four major steps: (1) Upload docking protein receptors. (2) Prepare chemical molecules for docking. The structural file is uploaded in 2D SDF format. (3) Run docking simulation. The docking simulation is carried out with machine learning system. (4) Acquire

molecular docking results including map information and pKd/pKi.

In vivo anti-acute myocardial infarction properties

Establishment of rat models

AMI model in rats with left anterior descending (LAD) coronary artery ligation as we have previously described (Raj *et al.*, 2017; Wang *et al.*, 2020). To reduce pain, all rats were anesthetized with isoflurane and ventilated artificially using a respirator. The AMI rat model was prepared by ligating a 6-0 silk suture with LAD 2 mm below the apex of the left atrial appendage. In the control group, rats were perforated but not ligated. After surgery, each rat was injected subcutaneously with 100,000 IU of penicillin to prevent infection and to increase the survival rate. The survival rate of both the sham group and the model group exceeded 90%.

Drug treatment

The control group received deionized water. Two weeks following the establishment of the AMI model, the AMI rats were randomized into three groups. The model group received deionized water, the captopril group received 3375 mg/kg of captopril, and the liquiritin group received 200 mg/kg of liquiritin. All rats were administered the intervention for four weeks. The captopril and liquiritin groups were gavaged once per day.

Measurement of hemodynamic indexes

Thirty minutes after the last treatment, all rats were sacrificed, and polystyrene was catheterized through the right carotid artery into the left ventricle of the heart. LVSP, LVEDP, +dp/dtmax, and -dp/dtmax were detected and analyzed by PowerLab (AD Instruments, Castle Hill, Australia).

Myocardial histopathology

Left ventricular myocardium containing myocardial infarction area was collected, and fixed with 4% paraformaldehyde. Thick tissue sections (4 μm) were prepared using paraffin-embedded tissues. Hematoxylin-eosin (HE) and Masson staining were used to observe the pathological changes.

Western Blotting

The concentration of myocardium-isolated total protein was determined using the BCA technique. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, blocked in 5% nonfat dry milk for 2 h, and incubated overnight. The proportions of antibodies were TLR4 (1:1000), MyD88 (1:1000), and NF-κB p65 (1:1000). After incubating the membranes overnight at 4°C, the secondary antibodies were administered for 2 h at room temperature. After washing three times with TBST, electrogenerated chemiluminescence (ECL) was employed to develop and fix the gels, and a gel imager (FluorChem M, ProteinSimple, USA) was utilized for photographing the gels and performing a semiquantitative analysis. The experiment was repeated three times. β-actin was used as an internal control.

Statistical analysis

All data were analyzed using SPSS 23.0, with a significance level at $P < 0.05$. Multiple groups were compared by one-way analysis of variance and the LSD method. GraphPad Prism 5.0 was applied to all statistical analyses.

Results

Molecular docking of liquiritin with the biological targets

The docking score (pKd/pKi) between liquiritin and TLR4 was 6.28, which was slightly lower than the native ligand (7.35) (Table 1 and Figure 1). And, the docking score between liquiritin and MyD88 was 5.73, which was higher than the native ligand (4.18) (Table 2 and Figure 2). The binding affinity between liquiritin and TLR4 or MyD88 is mainly intermolecular hydrogen bond. The findings of the molecular docking prediction indicated that liquiritin may function on the TLR4/MyD88 signaling pathway; however, *in vivo* animal investigations are required to verify these results.

Liquiritin improves heart function

LVSP, +dp/dt_{max}, and -dp/dt_{max} in the model group were decreased ($P < 0.01$), while LVEDP significantly increased compared with the sham group ($P < 0.01$). LVSP, +dp/dtmax, -dp/dtmax, and LVEDP in the liquiritin and captopril groups reversed the results compared to the model group ($P < 0.01$) (Figure 3). The results indicated that

Table 1. The docking scores (pKd/pKi) of liquiritin and TLR4.

Chemical constituents	Docking score	Binding of ligands to residues
Native ligand	7.35	Ile124, Glu122, Ser413, Phe151, Phe438, Phe126, Leu54, Val82, Leu87, Arg90, Phe121, Ile153
Liquiritin	6.28	Ile124, Ser413, Phe438, Leu54, Arg90, Phe121, **Ile52**

Bold means residue binding except for native ligand.

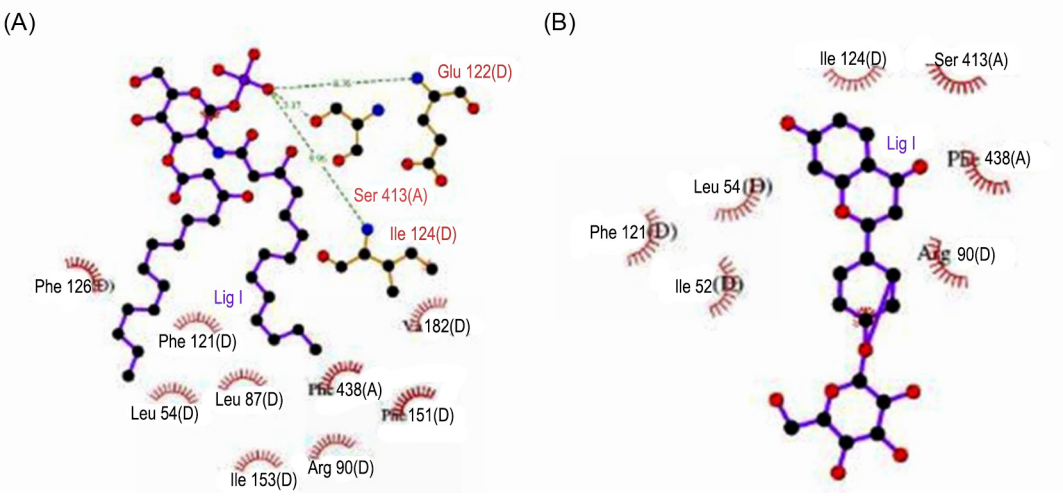

Figure 1. The pictures of native ligand and active site of TLR4. Native ligand (A, 2D), Liquiritin (B, 2D).

Table 2. The docking scores (pKd/pKi) of liquiritin and MyD88.

Chemical constituents	Docking score	Binding of ligands to residues
Native ligand	4.18	Tyr 257, Ala259, Thr277
Liquiritin	5.73	Tyr 257, Thr277, **Asp226**, **Ser224**, **Asp171**

Bold means residue binding except for native ligand.

liquiritin might enhance the cardiac function of rats with AMI.

Liquiritin improves the morphological changes via HE assay

As shown in Figure 4, the myocardium of rats in the sham group was evenly stained with normal morphology, clear texture, and orderly arrangement of myocardium cells, with a few cardiac fibroblasts. However, after ligation of LAD coronary artery induced AMI, pathological changes appeared in the myocardium, dyed unevenly, and were arranged in a disorderly manner. Myocardial cells in the model group were lytic, with fibroblasts proliferated and inflammatory cells infiltrated. Myocardial cells appeared with a regular cell arrangement and clear structure, and the pathological changes were attenuated in the liquiritin group and the captopril group.

Liquiritin improves the morphological changes via Masson staining

The myocardial fibers were red and collagen fibers were blue in each group. The texture of myocardial fibers was clear, arranged in an orderly manner, and the direction was consistent. A small amount of collagen fibers with uniform distribution could be observed in the sham group. Cardiac fibrosis was increased in the model group, while it was improved greatly in the captopril and liquiritin groups (Figure 5).

Effect of liquiritin on the expression of TLR4, MyD88, and NF-κB p65

Western blotting was used to measure the expression in the TLR4/MyD88/NF-κB signal pathway. As shown in Figure 6, the expression of TLR4, MyD88, and NF-κB p65 were significantly increased in the model group compared with the sham group ($P < 0.01$; $P < 0.05$). The expression of TLR4, MyD88, and NF-κB p65 were obviously decreased in the liquiritin group and the captopril group compared with the model group ($P < 0.01$; $P < 0.05$), but there were no significant differences between the liquiritin group and the captopril group.

Discussion

Myocardial inflammation plays a key role in the physiological and pathological mechanism of cardiac function and dysfunction. Myocardial inflammation is a general double-edged sword. Effective and appropriate inflammation is necessary and beneficial for host defense against injury. Excessive or chronic inflammation can cause severe myocardial damage to the myocardium, such as AMI (Liu *et al.*, 2019). AMI is a disease that causes damage and death to heart tissue due to the blockage of myocardial coronary arteries caused by atherosclerotic clots or arterial spasms, and now AMI has become one of the most common diseases that cause

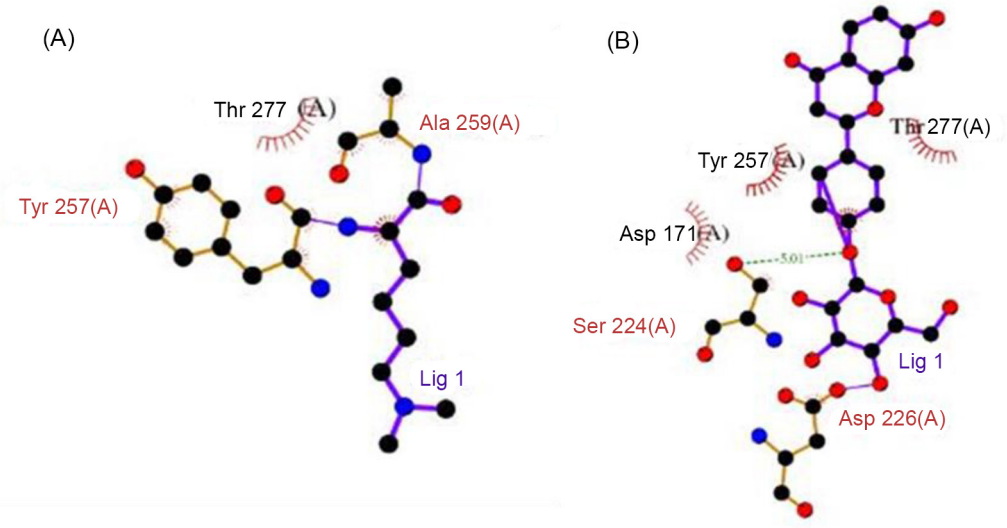

Figure 2. The pictures of native ligand and active site of MyD88. Native ligand (A, 2D), Liquiritin (B, 2D).

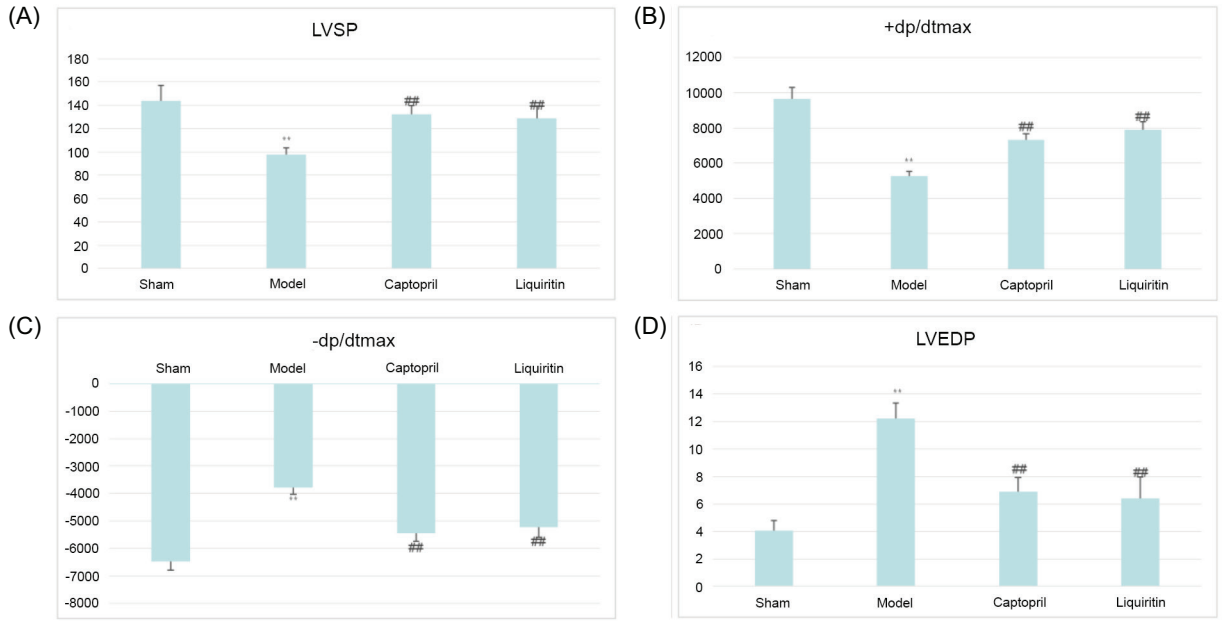

Figure 3. Liquiritin improves hemodynamics dysfunction on (A) LVSP; (B) +dp/dtmax; (C) -dp/dtmax; (D) LVEDP. The values are expressed as mean ± SD (n = 10). **P < 0.01 versus Sham group, ##P < 0.01 versus Model group.

morbidity and mortality worldwide. At present, the treatment of AMI mainly includes drug therapy, vascular reconstruction, and rehabilitation therapy, but these treatments have limited effect and find it difficult to prevent the progress of AMI (Amosse *et al.*, 2017). Although revascularization can effectively alleviate AMI, it is also accompanied by intractable complications, such as no-reflow after percutaneous coronary intervention (PCI), intrastent thrombosis, ischemia-reperfusion injury, etc. (Hernandez-Resendiz *et al.*, 2018). Therefore, it is of great significance for the development of new drugs to find effective therapeutic methods according to the pathogenesis of AMI. With the frequent and successful use of TCM in the prevention and treatment of AMI, the impact of Chinese medicine on AMI has drawn increasing attention.

As a "functional food," flavonoids can be widely used in the prevention and treatment of cardiovascular diseases. The anti-inflammatory effect of flavonoids can be used to prevent and treat CVDs found in fruits, vegetables, grains, bark, flowers, and tea (Choy *et al.*, 2019; Mozaffarian and Wu, 2018). Licorice contains multiple flavonoids, which possess a variety of biological activities.

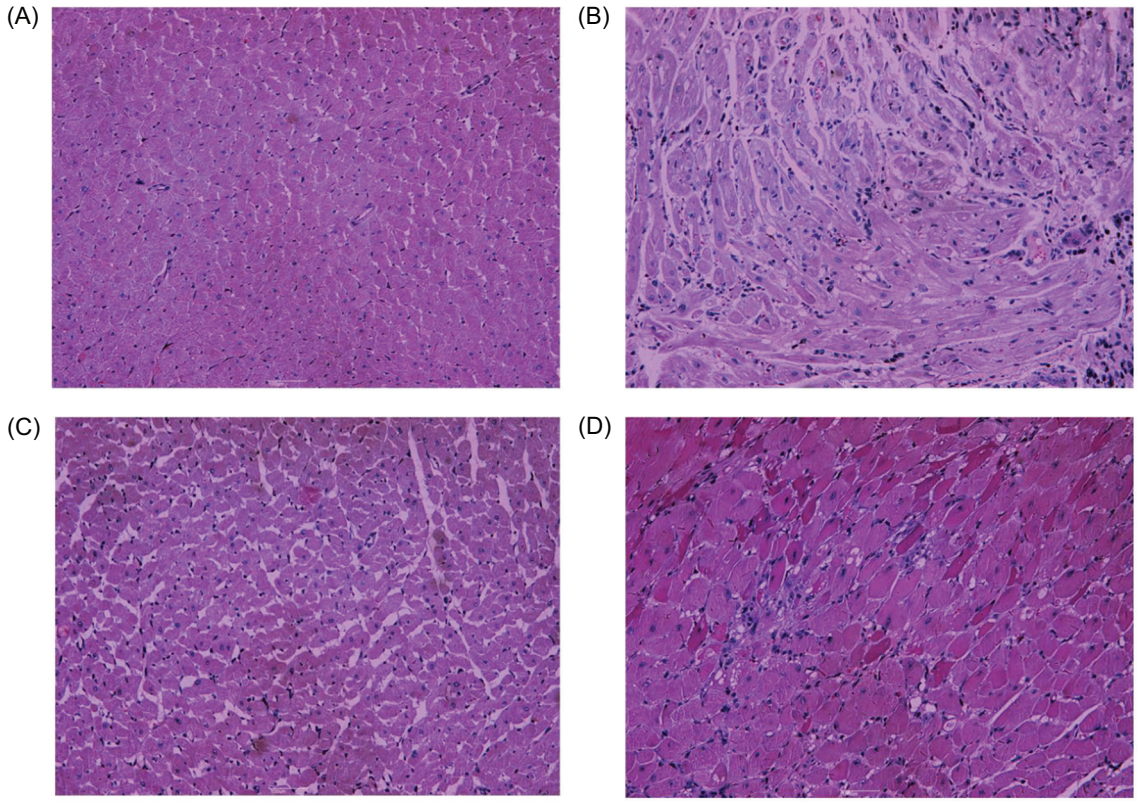

Figure 4. Morphological analysis of myocardial tissue stained with HE (×100). (A) Sham group. (B) Model group. (C) Captopril group. (D) Liquiritin group.

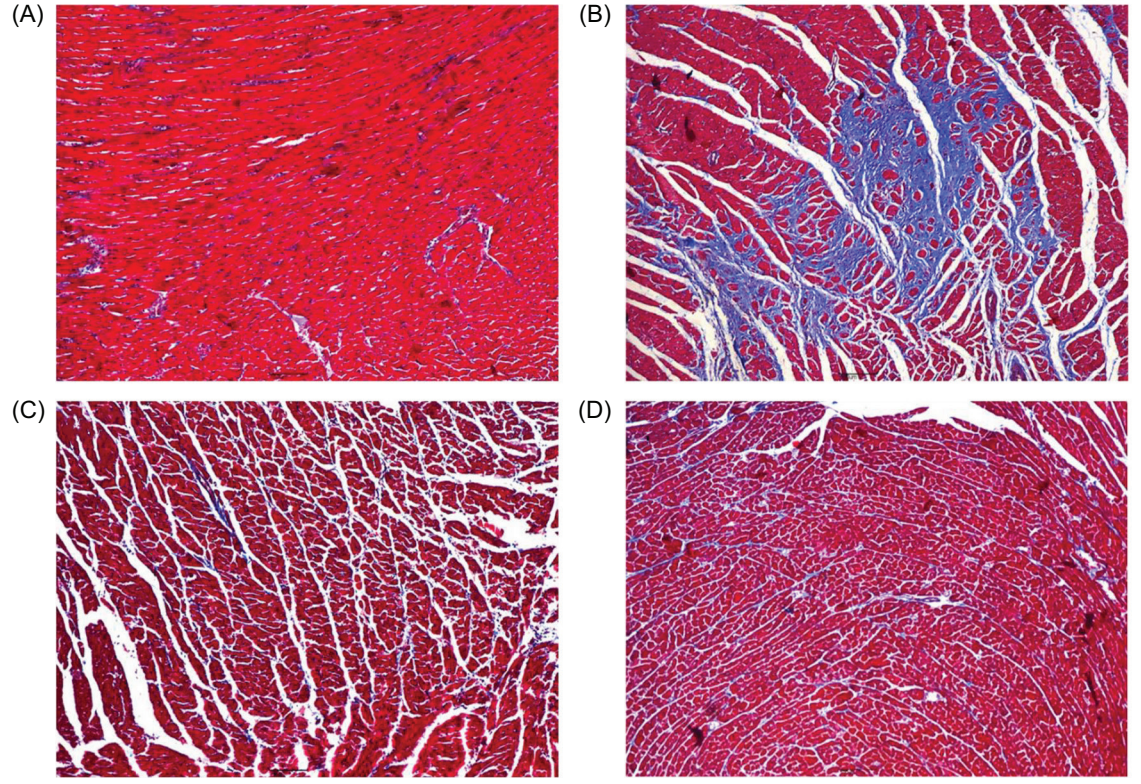

Figure 5. The Masson results of myocardium in different groups (×100). (A) Sham group. (B) Model group. (C) Captopril group. (D) Liquiritin group.

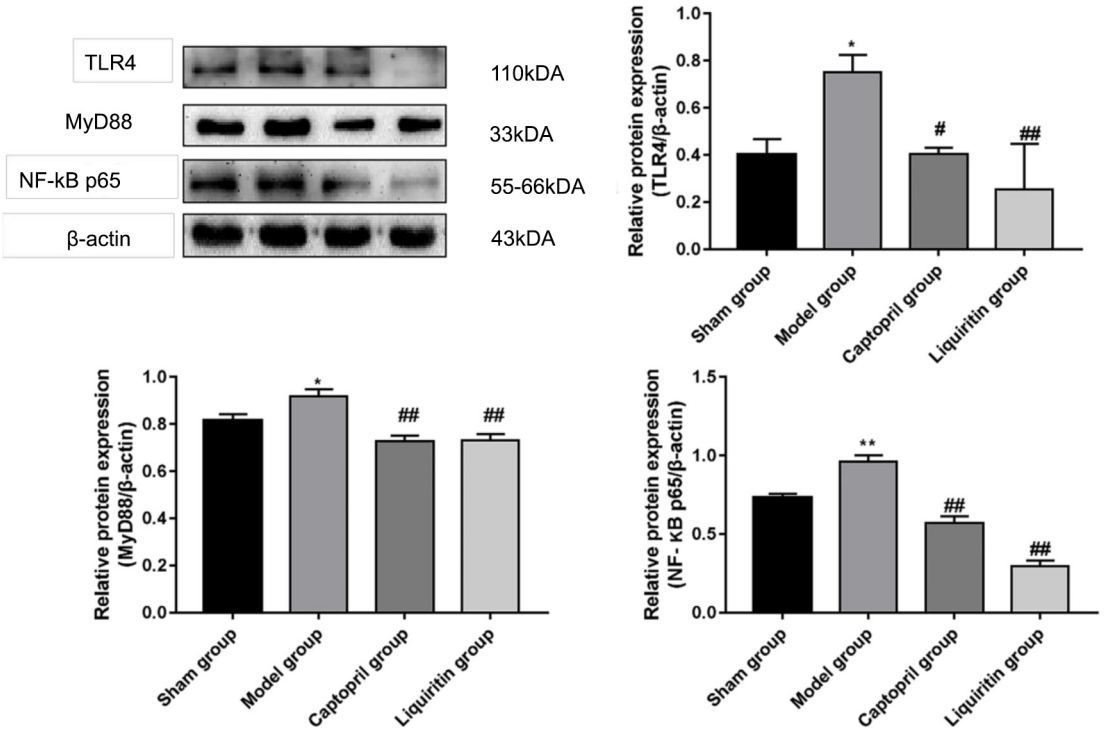

Figure 6. Effect of liquiritin on the expression of TLR4, MyD88, and NF-κB p65. The values were expressed as the mean±SD (n = 3), *P < 0.05, **P < 0.01, versus Sham group; #P < 0.01, ##P < 0.01, versus Model group.

Among them, liquiritin is a major constituent of Licorice, and it possesses anti-inflammatory activity. Liquiritin was effective in the inflammatory response to fructose stimulation *in vitro*, which can significantly reduce the release of inflammatory factors and NF-κB phosphorylation by inhibiting the IKKα/IκBα signaling pathway (Zhang *et al.*, 2016). Liquiritin can also significantly reduce the death rate of H9c2 cells after hypoxia/reoxygenation damage, increase the mitochondrial mass, and decrease the level of reactive oxygen species, and mitochondrial Ca^{2+} level (Thu *et al.*, 2021). The results of in vitro and in vivo experiments show that liquiritin can act as an agonist of AMP-activated protein kinase (AMPK), mainly because liquiritin can enhance the phosphorylation of AMPKα2 and decrease the phosphorylation of mTORC1, IκBα, and NFκB/p65 (Mou *et al.*, 2021).

In our study, liquiritin could increase the levels of LVSP, +dp/dtmax, and -dp/dtmax; reduce the level of LVEDP; and improve morphological changes through HE, and Masson staining, which showed that liquiritin has a good protective effect on AMI. Numerous studies have shown that TLR4 activates the expression of several pro-inflammatory cytokine genes that play a key role in myocardial inflammation, especially in myocarditis, myocardial infarction, ischemia-reperfusion injury, and heart failure (Hally *et al.*, 2017). Specifically, after TLR4 is stimulated by inflammatory signals, MyD88 binds to the cytoplasmic domain of TLR4 and activates IKK. Activated IKK kinase leads to phosphorylation and degradation of IκB in the proteasome, and NF-κB is then released from the NF-κB complex and transported to the nucleus, resulting in gene expression of pro-inflammatory cytokines (Yang *et al.*, 2016). Activation of TLR4/MyD88 signaling pathway leads to direct activation of NF-κB and promotes secretion of pro-inflammatory cytokines. In this study, liquiritin decreased the expression of TLR4, MyD88, and NF-κB p65 proteins, suggesting that liquiritin inhibited AMI through TLR4/MyD88/NF-κB signaling pathway.

However, the current study has several limitations. First of all, this study only adopted an *in vivo* experiment, without further verification by an *in vitro* cell experiment. Secondly, this study lacked the use of TLR4 inhibitor, so as to better explore the mechanism of action of liquiritin on AMI.

Conclusion

In conclusion, molecular docking combined with *in vivo* evaluation showed that liquiritin has a cardioprotective effect on AMI model rats by inhibiting the TLR4/MyD88/NF-κB signaling pathway. This pathway may be a new

potential therapeutic target of liquiritin in the treatment of AMI, and these properties of liquiritin can be further explored to develop viable anti-AMI agents.

Declarations

Funding

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Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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