



Potential mechanisms of Qili Qiangxin capsule to prevent pulmonary arterial hypertension based on network pharmacology analysis in a rat model

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Background: Qili Qiangxin capsule (QQC), a traditional Chinese medicine, has recently been approved to treat pulmonary arterial hypertension (PAH). However, the multi-target mechanism through which QQC acts on PAH has not been clarified. The objective of this study was to explore the pharmacological processes of QQC for treating PAH.

Methods: The rat model of PAH was established by administering monocrotaline (MCT). The impact of QQC on PAH was studied in treatment group that received QQC orally over a period of 4 weeks. The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database was searched for active compounds and QQC targets that were then identified and downloaded. Then, PAH-related targets were obtained from five databases [GeneCards, DrugBank, Online Mendelian Inheritance in Man (OMIM), Therapeutic Target Database (TTD), and PharmGKB]. The QQC targets for PAH were compiled after they had been overlapped with one another. Furthermore, the STRING network platform, the Cytoscape tool, networks of protein-protein interaction (PPI) were used, and core target analyses were carried out. Moreover, molecular docking techniques were employed in this research. Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment studies of overlapping targets were carried out using the R software (version: 4.0.5; Lucent Technologies Co., Ltd., China). Finally, we verified the synergistic action mechanisms using western blotting and immunofluorescence analysis on PAH rats who were treated with or without QQC.

Results: The search of the TCMSP database showed that there were 11 active ingredients in QQC that treated PAH. PPI network showed that *AKT1*, *TP53*, *JUN*, and *MAPK1* were the most important targets in the treatment of PAH. Moreover, Molecular docking techniques showed that the affinity between the bioactive compounds in QQC and their PAH targets was strong. *In vivo* experiments demonstrated that QQC may attenuate the progression of MCT-stimulated PAH in rats. Furthermore, the protective effect was mediated by inhibiting the PI3K/AKT pathway. The active compounds mainly included quercetin, kaempferol, formononetin, and luteolin, which had good docking scores and targeted the AKT protein.

Conclusions: QQC might activate the PI3K/AKT signaling pathway to ameliorate MCT-induced PAH. These findings support the clinical use of QQC and provide the foundation for further studies.

Keywords: Molecular docking; network pharmacology; pulmonary arterial hypertension (PAH); Qili Qiangxin capsule (QQC)

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Introduction

Pulmonary arterial hypertension (PAH) is a complex and lethal disorder characterized by remodeling of smaller pulmonary arteries and vascular proliferation that leads to right ventricular failure or death (1). At present, the first-line treatments for PAH mainly include endothelin receptor antagonist, the phosphodiesterase type-5 inhibitors, soluble guanylate cyclase agonist, calcium antagonist, prostacyclin receptor agonist. However, some western medications have been reported to be associated with numerous side effects and adverse reactions (2). Therefore, more feasible and safer therapeutic strategies are required for PAH management. In recent years, several exploratory studies demonstrated that THM, particularly some active components derived from THM (i.e., quercetin, baicalin, ursolic acid and berberine), could be used as therapeutic candidates to prevent or cure PAH (3-6).

Qili Qiangxin capsule (QQC) is a well-known traditional Chinese medicine formulation comprising 11 herbs that is commonly used to treat heart failure (7,8). Nonetheless, its precise targets remain unclear. It is thought to work through a variety of mechanisms, including decreasing the remodeling of cardiac fibrosis, enhancing the function of the heart, and modulating inflammation (9,10).

In view of this, the objective of the present research was to identify the fundamental active constituents of QQC and possible pharmacological pathways that contribute to its anti-pulmonary hypertension functions. In addition, *in vivo* experiments were carried out to validate the results in this article. Meanwhile it is also the first time that network pharmacology and experimental validation were conducted to explore the mechanism of QQC on PAH therapy. *Figure 1* depicts the overall design of the present research and its workflow. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-901/rc>)

Methods

Identification of active ingredients and target proteins

The active ingredients of QQC were determined by using the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database and analysis platform (11). The rate and degree of absorption of medicine in the human bloodstream are referred to as oral bioavailability (OB). Drug-like (DL) features describe the properties of a drug with a

specified functional group or physical characteristics that are the same or comparable. To evaluate the transportation and absorption of drugs within the intestinal epithelial cells, the human intestinal cell line Caco-2 was used. The half-life of a medication is an essential measure for determining the dosage interval, the dosage that patients receive, and drug buildup since it represents the proportion of drugs in the bloodstream or the body (12). To identify those ingredients with the highest activity were subsequently tested if their OB was greater than 30% and their DL features were greater than 0.18. The SMILE configurations of the components were also acquired from the PubChem database.

Possible active ingredients targets and PAH

The ingredient-related targets were estimated with the aid of the TCMSP database. On 30 May 2021, five commonly used databases, namely the PharmGKB, GeneCards, Online Mendelian Inheritance in Man (OMIM), Therapeutic Target Database (TTD), and DrugBank database were used to determine the known PAH treatment targets. Several ingredient-related targets were identified as genes associated with PAH after duplicate entries were eliminated. After an intersection of QQC and PAH targets was found, the possible targets associated with both active components and PAH were identified. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Protein-protein interaction (PPI)

As described in a previous study, a PPI analysis was carried out to search for important protein targets exhibiting therapeutic benefits against PAH (13). A database called STRING (<https://cn.string-db.org/>, downloaded on 30 May 2021) was used to investigate possible targets associated with both components and PAH. Four kinds of confidence ratings were used to assess PPI, namely low (0.1), medium (0.4), high (0.7), and highest (0.9). To facilitate subsequent investigations, prospective therapeutic targets exhibiting scores ≥ 0.9 were chosen.

Establishment and analysis of a network

Using the Cytoscape 3.8 program, a network of ingredient-target relationships was created to investigate the interaction between components and their possible therapeutic targets.

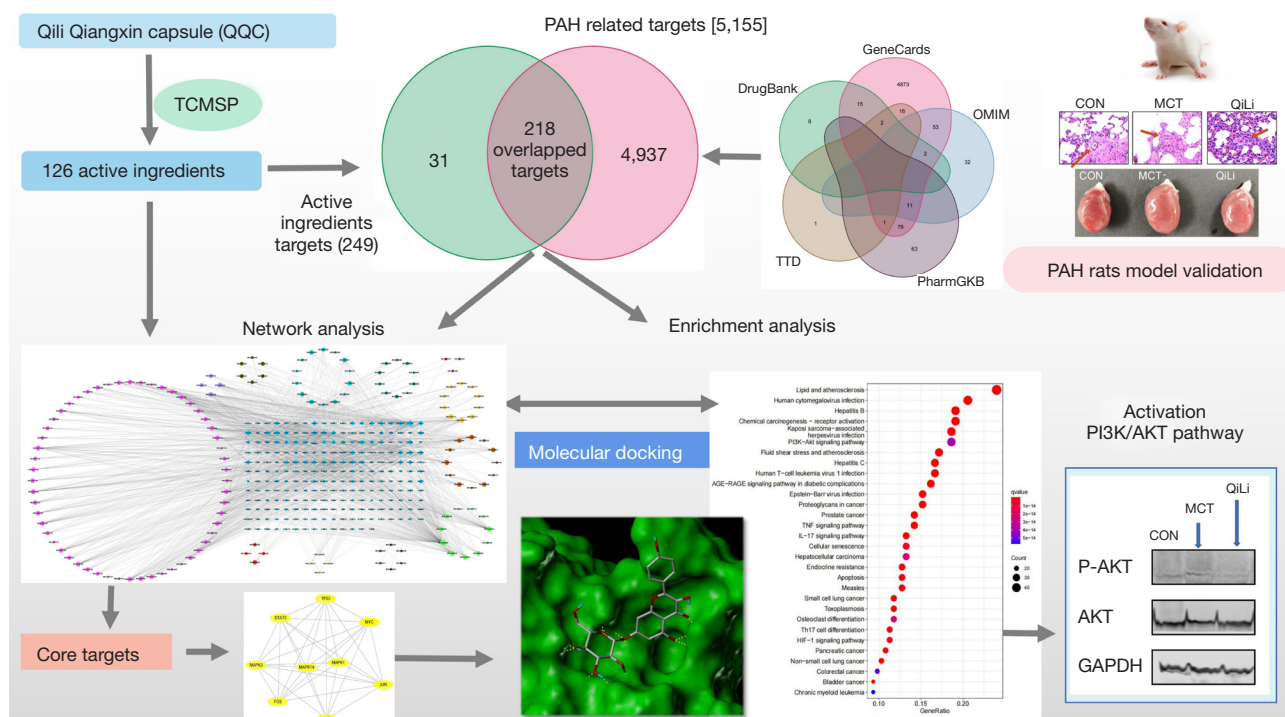


Figure 1 A flowchart showing the underlying mechanisms by which QQC prevents PAH by a network pharmacology approach and PAH rats model validation. Red arrows show the pulmonary arteriole WT by H&E staining ($\times 200$) in each group rats. QQC, Qili Qiangxin capsule; TCMSP, Traditional Chinese Medicine Systems Pharmacology; OMIM, Online Mendelian Inheritance in Man; TTD, Therapeutic Target Database; CON, control group; MCT, monocrotaline; QiLi, MCT + QQC group; PAH, pulmonary arterial hypertension; WT, wall thickness; H&E, hematoxylin and eosin.

In order to determine the relevance of nodes within the network, several of these analytic variables including degree were determined with the aid of the Network-Analyzer software. In the case where a node's extent value exceeds twice the mean level of all nodes, it is considered to be an essential element of the network.

Enrichment analysis on possible treatment target

All possible treatment targets were input into the DAVID system (<https://david.ncicrf.gov/>, v6.8), and we subsequently performed online Gene Ontology (GO) term enrichment and the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis to delve into their biological activities. Moreover, according to the gene ratio and the P value, the predominant signaling pathways and critical proteins involved in biological processes were determined.

Validation of essential active compounds and target interactions using molecular docking

Molecular docking analysis of the predominant active components of QQC and possible treatment targets was performed with the SYBYL-X software (version: 2.1.1; Tripos Company, USA) to measure the accuracy of the GO and KEGG enrichment analyses. X-ray crystallography was used to distinguish the crystal protein structures, which were then uploaded into SYBYL-X 2.1.1 and docked with the active compounds of QQC. To assess the outcomes, the docking metric DockScore was used.

Animal model and ethics approval

In the present research, a rat model was used (14). Animals were procured from the SLAC Experimental Animal Center in Shanghai, including 8-week-old Sprague Dawley

males measuring 230–250 grams. The ethics committee of Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine (Shanghai, China) granted approval for the experiment procedure (No. 81603099). Moreover, all procedures were performed in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

In an ecologically closed chamber with a temperature ranging between 20–26 °C and a humidity range of 50%±5%, all rats were subjected to a 12-hour darkness/light cycle, and they had an unrestricted supply of water and food. Following 1 week of acclimatization, 24 rats were classified into three groups using a random classification: (I) the control group (n=8) was treated with isotonic sodium chloride solution; (II) the monocrotaline (MCT) group (n=8) was treated with MCT at 60 mg/kg; and (III) the QiLi group (n=8) was treated with MCT + QQC at 1 g/kg/day. The PAH model was induced as described previously (14). The control group rats received a subcutaneous injection of isotonic sodium chloride solution on day 1, followed by 4 weeks of water. The MCT group and QiLi group rats were injected subcutaneously with MCT (60 mg/kg) in the ventral thorax on day 1; based on previously reported method, drug powder was dissolved in sterile water at a concentration of 0.1 g/mL for QiLi group, and then, rats in QiLi group were administered by gavage (1 g/kg) once a day during the 4 weeks (15).

Hematoxylin and eosin (H&E) staining and western blotting

The rats were weighed and anesthetized intraperitoneally with sodium pentobarbital (30 mg/kg), followed by euthanasia by an intraperitoneally injection of sodium pentobarbital at a dosage of 150 mg/kg. The left lung tissues were sliced for histological and immunohistochemistry investigations, and the right lung tissues were frozen at –80 °C for western blot assay. Paraformaldehyde (4%) was used to fix the left lungs that had been isolated at room temperature for 48 hours before paraffin embedding and slicing of the tissues at a thickness of 4 µm. The lung tissue slices were subjected to staining with H&E in accordance with the instructions provided by the manufacturer. Microscopy was used to investigate the pulmonary arteries' structural remodeling. In the present study, proteins were isolated from lung tissues by homogenizing them in lysis buffer in combination with phosphatase blockers (Cell

Signaling Technology, Inc., Danvers, MA, USA) and enzyme protease (Beyotime Institute of Biotechnology, Haimen, China). The BCA Protein Assay kit was used to assess the percentage of protein in the sample. A comparable quantity (50 g) of protein lysate was isolated using 10% SDS-PAGE and subsequently loaded onto a polyvinylidene difluoride membrane for further analysis and characterization. In addition, blocking of the membranes was conducted for 2 hours at ambient temperature using 5% bovine serum albumin, followed by incubation with anti-GAPDH (1:1,000; Cell Signaling Technology 5174), anti-p-AKT473 antibody (1:1,000; Cell Signaling Technology 4060S), and anti-AKT antibody (1:1,000; Cell Signaling Technology 9272S) throughout the night at 4 °C. Furthermore, TBST was used to wash the membranes, followed by incubation with IDye800CW conjugated secondary antibody (1:10,000; Cell Signaling Technology 5151) for 1 hour at ambient temperature. Protein bands were captured by the Odyssey imaging system (LICOR).

Statistical analysis

We used GraphPad Prism 5 software (GraphPad Software, Inc., LA Jolla, CA, USA) to perform statistical analyses on the obtained data. The data were expressed as the mean ± standard error of the mean. Results were considered statistically significant if $P < 0.05$.

Results

Screening for bioactive components and QQC targets relevant to PAH therapy

First, the TCMSP database was used to screen 154 active compounds. Specifically, 126 active compounds were obtained as essential components as their OB and DL values were above the average. An additional study was conducted on 249 targets associated with the 126 active compounds. In addition, 5,155 targets for PAH treatment were obtained from TCMSP, DrugBank, OMIM, TTD, and PharmGKB (*Figure 1*). Using a Venn diagram to map QQC- and PAH-related targets (*Figure 2A,2B*), we identified 218 intersecting targets and their associated bioactive components. Moreover, Cytoscape was used to visualize 126 bioactive ingredients and 218 intersecting targets, which showed the compounds in QQC that were targets for treating PAH (*Figure 2C,2D*).

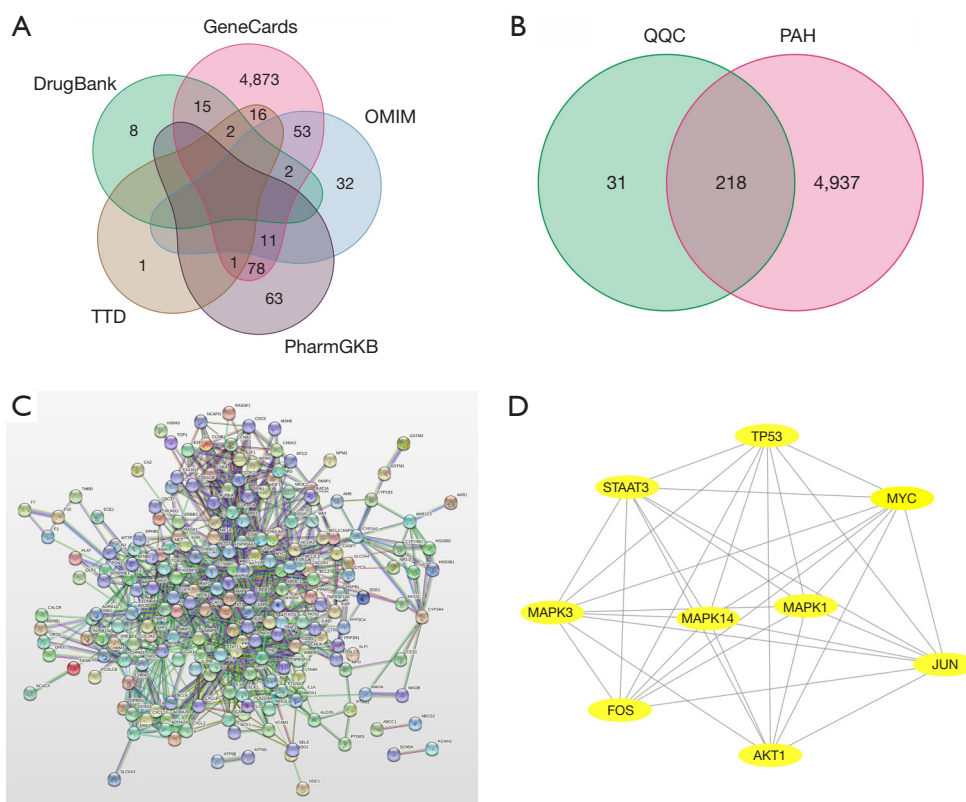


Figure 2 Screening and prediction of key ingredients and targets using network pharmacology. (A) PAH-related targets obtained from PharmGKB, GeneCards, OMIM, TTD, and DrugBank databases. (B) The potential targets connected to both active ingredients and PAH. (C) A PPI network from STRING database. (D) Core targets. OMIM, Online Mendelian Inheritance in Man; TTD, Therapeutic Target Database; QQC, Qili Qiangxin capsule; PAH, pulmonary arterial hypertension; PPI, protein-protein interaction.

Establishment of a PPI network and identification of core targets

To investigate the treatment mechanism of QQC for PAH, a PPI network was established by inputting 218 intersecting targets into the STRING platform. The average node degree of the target network comprising 218 nodes as well as 61 edges was 8.64. In Cytoscape, the PPI network data generated in the STRING database was loaded, and the highest nine core targets were identified using the cytoHubba plug-in in decreasing order of degree (Figure 3). *AKT1*, *JUN*, *MAPK1*, and *TP53* received the maximum possible ratings, therefore results showed that these may perform critical functions in the treatment of PAH.

Enrichment analysis of GO and KEGG

The key biological activities and pathways were identified

with GO and KEGG enrichment studies carried out using the R software. To treat PAH, findings showed that the functions of QQC were correlated with the reaction to oxidative stress, the responsiveness to medications, and the cellular response to chemical stress. Figure 4A shows a list of sample GO terms in a graphical format. The KEGG pathway enrichment analysis in the present research focused on atherosclerosis and lipid pathways, as well as the AGE-RAGE signaling pathway in hepatitis B and diabetes-related complications, all of which are depicted in Figure 4B.

Molecular docking

Using SYBYL-X 2.1.1 software, a total of 26 molecule-target pairings were identified and verified using molecular docking. Consequently, 6 molecule-target pairs, which were quercetin/*MAPK1*, quercetin/*JUN*, luteolin/*MAPK1*, kaempferol/*JUN*, baicalein/*TP53*, and kaempferol/*AKT1*,

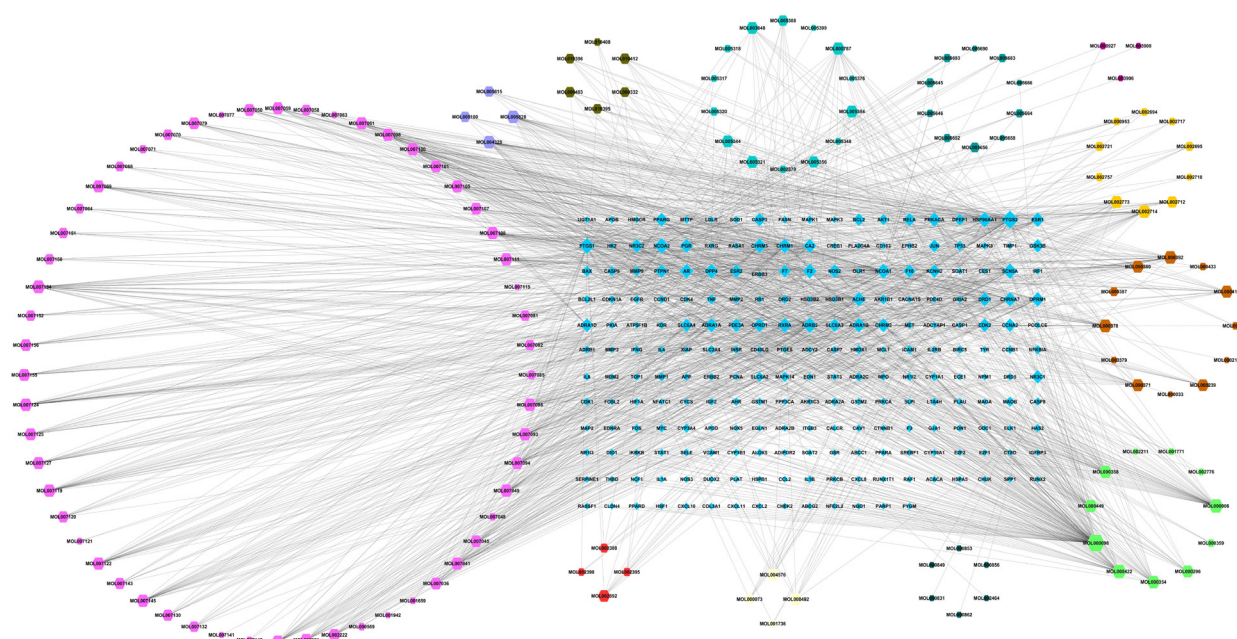


Figure 3 A network constructed from Cytoscape analysis results highlighting the interactions between the compounds of QQC and overlapped targets from PAH and QQC. Blue diamonds represent overlapped targets and are arranged in rectangles. The screened compounds of 11 herbs from QQC were arranged in circles, with each circle representing potent compounds in each herb. The degree to which the compound interacts with the target is visualized by the size of the tag. QQC, Qili Qiangxin capsule; PAH, pulmonary arterial hypertension.

demonstrated a strong binding association, as presented in *Table 1*. *Figure 4C,4D* depicts 3D molecule docking images of kaempferol/AKT pairs.

Histological evaluation and western blotting

We successfully established a rat model of PAH by injecting MCT subcutaneously. QQC was shown to be effective in suppressing MCT-induced pulmonary vascular remodeling. In the present research, we used H&E staining to quantify the small pulmonary arteries to assess pulmonary vascular remodeling. Following MCT therapy, the indicators of wall thickness (WT) of pulmonary arterioles in the MCT group were considerably higher as opposed to the ones of the control group ($P < 0.01$). QQC (1 g/kg) treatment substantially suppressed these pathological alternations in the lungs as opposed to the MCT treatment (*Figure 5*). We identified several genes that may interact with QQC through. Moreover, we investigated whether QQC had an effect on the PI3K/AKT signaling pathway. The PI3K/AKT pathway is an essential proliferation-related signaling pathway. In this regard, we investigated the expression of

AKT, which is a protein that performs a critical function in the PI3K/AKT signaling pathway. MCT substantially elevated the p-AKT phosphorylation levels of AKT in lung tissues in contrast with the control group ($P < 0.01$). After the administration of QQC, the elevated phosphorylation of AKT was dramatically attenuated ($P < 0.01$). Overall, QQC substantially attenuated the expression of phosphorylated AKT as well as the proportion of p-AKT/AKT induced by MCT ($P < 0.01$).

Discussion

Heart failure is highly prevalent in patients with PAH, especially those who have had the disease for a long time. A growing body of research shows that heart failure patients might benefit from the application of QQC in combination with conventional Western treatment, which has the potential to enhance treatment effectiveness and improve the quality of life of patients (16). Nevertheless, the material foundation of QQC and the mechanisms through which it can treat PAH are still being investigated.

Traditional Chinese medicine prescriptions are a

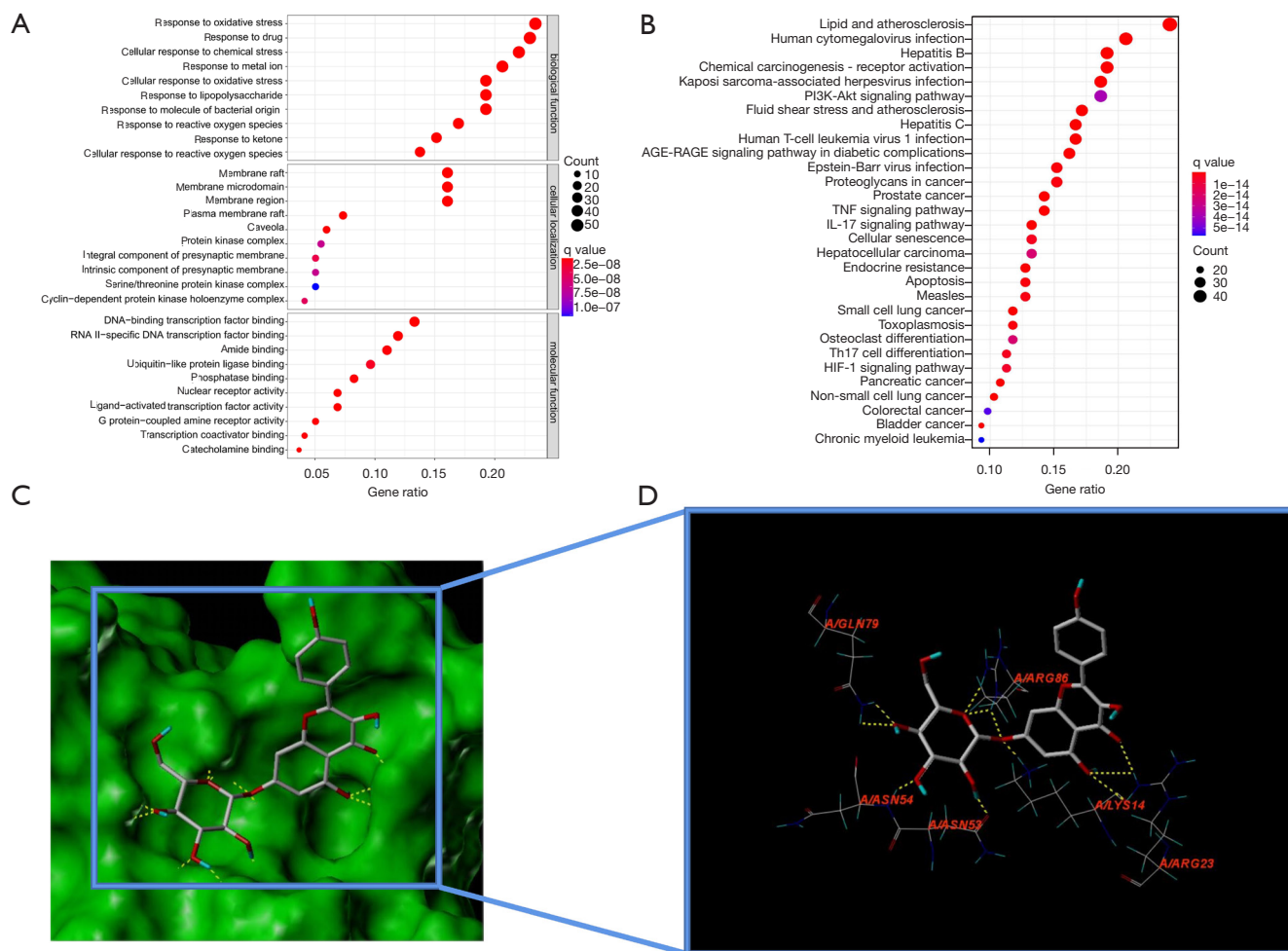


Figure 4 GO term and KEGG enrichment analysis and molecular docking experimental validation of acacetin binding core targets predicted by network pharmacology. (A,B) GO term and KEGG enrichment analysis. (C,D) A 3D molecular docking model of Kaempferol/ AKT pairs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table 1 List of the scores of the molecular docking with potential compounds and key targets proteins

Molecular name	Targets	Protein data bank ID	Docking score (kcal/mol)
Quercetin	MAPK1	6SLG	6.1728
Quercetin	JUN	6Y3V	5.6552
Luteolin	MAPK1	6SLG	5.628
Kaempferol	JUN	6Y3V	5.3342
Baicalein	TP53	3LW1	5.2171
Kaempferol	AKT1	1UNQ	5.521

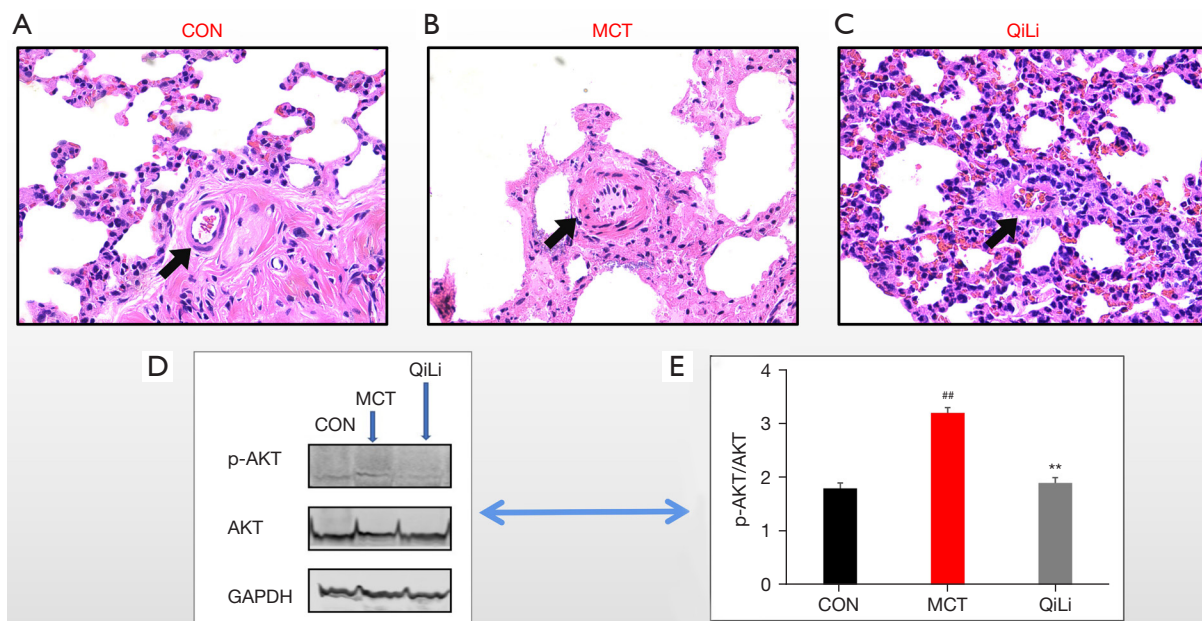


Figure 5 PAH rats model validation. H&E staining of lung tissue ($\times 200$) in rat model. Black arrows show the pulmonary arteriole WT in each group rats. (A) Control group. (B) MCT group. (C) QiLi group. (D,E) The expression levels of AKT and phosphorylated AKT assessed via western blotting. The data are expressed as the mean \pm SEM ($n=8$). ** $P<0.01$ compared with the control group; ## $P<0.01$ compared with the MCT group. CON, control group; MCT, monocrotaline; QiLi, QQC + MCT group; PAH, pulmonary arterial hypertension; H&E, hematoxylin and eosin; WT, wall thickness; SEM, standard error of mean.

complicated system that involves a broad variety of molecular targets and mechanisms. This makes it difficult to elucidate their effective constituents, therapeutic targets, and pharmacological mechanisms (17). Network pharmacology is an emerging discipline based on the theory of systems biology, which can accurately decipher the associations among the drugs, targets, and diseases at a systematic and comprehensive level (18). Increasing evidence supports the reliability of the network pharmacology method, which may be an effective way to study the pharmacological mechanisms of Traditional Chinese medicine (19).

In this study, Luteolin, kaempferol, baicalein, formononetin, and quercetin networks within QQC all had greater degree values calculated by Cytoscape software as opposed to the other components, and these critical components were all shown to impact the four hub targets, which were *MAPK1*, *JUN*, *TP53*, and *AKT1*. Moreover, molecular docking analysis showed that these 5 essential components had the highest binding affinity, suggesting that they could perform an integral function that enables QQC to reduce pulmonary hypertension. Kaempferol can prevent and reverse ventricular fibrosis and cardiac dysfunction by suppressing the production of inflammatory apoptosis

and cytokines, enhancing antioxidant transcription factors, and promoting the generation of nitric oxide from the vascular endothelium (20–22). Quercetin can alleviate PAH by inhibiting cardiac fibrosis and balancing cell proliferation and apoptosis (23–25). Luteolin can protect against lipopolysaccharide (LPS)-induced cardiomyocyte hypertrophy and autophagy in rat cardiomyocytes (26). In addition, baicalein and formononetin may prevent PAH.

The KEGG pathway enrichment analysis showed that a number of pathways were significantly correlated with the PAH pathological process. In general, the most significant pathways involved were atherosclerosis, PI3K/AKT, HIF-1, TNF, AGE-RAGE, and IL-17. A significant interaction between the PI3K/AKT signaling pathway and other proliferation-related signaling pathways was observed among these pathways (27). Furthermore, earlier research has shown that the progression of PAH is strongly correlated with abnormal transduction of the PI3K/AKT signaling pathway (28). Activation of the PI3K/AKT signaling pathway in hypoxic environments or in rats experiencing MCT-induced PAH results in the thickening of the pulmonary vascular medial wall, proliferation, and apoptosis of smooth muscle cells of the pulmonary artery in humans (29).

In recent years, network pharmacology analysis was applied in a few studies to explore single herbal components of Traditional Chinese medicine involved in the treatment of PAH. Chen *et al.* (30) reported that the anti-PAH effect of genistein may be closely related to peroxisome proliferator-activated receptor γ (PPAR γ), apoptotic signaling pathway, and the nitric oxide synthesis process. The results of another research showed that the effect of resveratrol may be closely associated with targets such as AKT1, MAPK3, SIRT1 and SRC, as well as biological processes such as cell proliferation, inflammatory response, and redox balance (31). However, the above studies lacked experimental verification. Therefore, we decided to investigate the PI3K/AKT signaling pathway. We further illustrated that QQC reduced the relative expression of proteins for p-AKT/AKT in the local lung tissue of PAH rats *in vitro*. Finally, we observed that QQC ameliorated MCT-induced PAH, at least in part, by inhibiting the PI3K/AKT signaling pathways through its antiproliferative effects.

These findings indicated that five candidate compounds are a promising source of anti-PAH agents. Furthermore, molecular docking analysis suggested that the active compounds interacted strongly with the PI3K/AKT signaling proteins. This study is a preliminary attempt to explore the effects of active compounds on the PI3K/AKT pathway. The results of animal experiments were consistent with the results of network pharmacology mining, which verified the reliability of network pharmacology prediction and confirmed the accuracy of QQC in the treatment of PAH.

Conclusions

Our research findings indicated that QQC suppressed pulmonary arterial smooth muscle hyperplasia and lowered the level of expression of p-AKT/AKT proteins. However, QQC can act on multiple pathways and has multiple biological functions, such as immunomodulation, anti-hypoxia, and anti-inflammation. Hence, more well-designed animal research, *in vitro* test models, and human trials are necessary to better reveal the mechanisms by which QQC can treat heart disease.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-901/rc>

Data Sharing Statement: Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-901/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-901/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The ethics committee of Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine (Shanghai, China) granted approval for the experiment procedure (No. 81603099). Moreover, all procedures were performed in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

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