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## Clinical and Translational Research

## Exploring the pharmacological mechanism of Wuzhuyu decoction on hepatocellular carcinoma using network pharmacology

Jia-Ying Ouyang, Wei-Jie Lin, Jia-Mei Dong, Yang Yang, Hai-Kui Yang, Zhi-Ling Zhou, Rui-Qi Wang

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Wuzhuyu decoction, a traditional Chinese medicinal formula, is effective in treating hepatocellular carcinoma (HCC).

**AIM**

To explore the potential mechanism of action of Wuzhuyu decoction against HCC.

**METHODS**

The active components of each Chinese herbal medicinal ingredient in Wuzhuyu decoction and their targets were obtained from the Traditional Chinese Medicine Database and Analysis Platform. HCC was used as a search query in GeneCards, Online Mendelian Inheritance in Man, Malacards, DisGeNET, Therapeutic Target Database, and Comparative Toxicogenomics Database. The overlapping targets of the Wuzhuyu decoction and HCC were defined, and then protein-protein interaction, Gene Ontology, and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were performed. CytoHubba was used to select hub genes, and their binding activities and key active components were verified using molecular docking.

**RESULTS**A total of 764 compounds, 77 active compounds, and 204 potential target genes were identified in Wuzhuyu decoction. For HCC, 9468 potential therapeutic target genes were identified by combining the results from the six databases and removing duplicates. A total of 179 overlapping targets of Wuzhuyu decoction and HCC were defined, including 10 hub genes (tumor necrosis factor, interleukin-6, *AKT1*, *TP53*, caspase-3, mitogen-activated protein kinase 1, epidermal growth factor receptor, *MYC*, mitogen-activated protein kinase 8, and *JUN*). There were six main active components (quercetin, kaempferol, ginsenoside

Rh2, rutaecarpine,  $\beta$ -carotene, and  $\beta$ -sitosterol) that may act on hub genes to treat HCC in Wuzhuyu decoction. Kyoto Encyclopedia of Genes and Genomes enrichment analysis mainly involved the mitogen-activated protein kinase, p53, phosphatidylinositol-4,5-bisphosphate 3-kinase-Akt, Janus kinase-signal transducer of activators of transcription, and Hippo signaling pathways. Further verification based on molecular docking results showed that the small molecule compounds (quercetin, kaempferol, ginsenoside Rh2, rutaecarpine,  $\beta$ -carotene, and  $\beta$ -sitosterol) contained in Wuzhuyu decoction generally have excellent binding affinity to the macromolecular target proteins encoded by the top 10 genes.

## CONCLUSION

This study revealed that Wuzhuyu decoction may be a latent multicomponent, multitarget, and multipathway treatment for HCC. It provided novel insights for verifying the mechanism of Wuzhuyu decoction in the treatment of HCC.

**Key Words:** Wuzhuyu decoction; Hepatocellular carcinoma; Network pharmacology; Pharmacological mechanism; Bioinformatics

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**Core Tip:** Six main active components (quercetin, kaempferol, ginsenoside Rh2, rutaecarpine,  $\beta$ -carotene, and  $\beta$ -sitosterol) and ten core genes (tumor necrosis factor, interleukin-6, *AKT1*, *TP53*, caspase-3, mitogen-activated protein kinase 1, epidermal growth factor receptor, *MYC*, mitogen-activated protein kinase 8, and *JUN*) were obtained from Wuzhuyu decoction. In addition, 179 pathways were obtained from Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis, which indicated that Wuzhuyu decoction is a multicomponent, multitarget, and multipathway treatment for hepatocellular carcinoma. The active components of Wuzhuyu decoction may participate in these pathways *via* the top 10 hub genes, which achieves the therapeutic effect on hepatocellular carcinoma.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world, ranking sixth in the global mortality rate of malignant tumors. It is the most common pathological type of primary liver carcinoma, accounting for approximately 75%-85% of all cases[1]. The etiology and pathogenesis of HCC have not been defined; however, it is generally believed to be related to cirrhosis, viral hepatitis[2], excessive alcohol consumption[3], and chemical carcinogens such as aflatoxins[4]. Presently, the treatment method for HCC is relatively simple. If patients are diagnosed and treated early, they have a good prognosis through surgery and chemotherapy. However, the invasion of HCC is inconspicuous. The majority of the patients are diagnosed late, losing the best opportunity to undergo surgery and therefore only receive drug maintenance therapy[5]. Clinical first-line drugs for treating HCC include sorafenib and lenvatinib[6]. These drugs are expensive and impose heavy family and socioeconomic burdens. In addition, adverse drug reactions and tolerance often occur in clinical practice. Therefore, it is necessary to search for other effective, safe, and inexpensive drugs to treat HCC.

Traditional Chinese medicine has been practiced for thousands of years to treat various diseases[7]. For some patients with HCC who are unable to receive surgical treatment, whose bodies develop resistance to targeted drugs, or whose families cannot afford the relatively high cost of clinical treatment, traditional Chinese medicine treatment is selected to improve their quality of life and prolong life. Wuzhuyu decoction, as a warming interior formula, can warm the middle jiao (stomach, liver, spleen, and gallbladder) and tonify deficiencies from the Treatise on Febrile Diseases. Wuzhuyu decoction usually consists of four herbs: Wuzhuyu (*Evodiae Fructus*), Renshen (*Panax ginseng* C. A. Mey.), Shengjiang (*Zingiber officinale* Roscoe), and Dazhao (*Jujubae Fructus*). According to traditional Chinese medicine, Wuzhuyu plays a role in dispersing cold and relieving pain, stopping diarrhea, and relieving vomiting[8].

Recent studies have reported that it also exhibits anti-inflammatory and antitumor activities, induces cell cycle arrest, and suppresses tumor cell migration[8-10]. Renshen and Dazhao can boost immune function[11,12]. Ginsenoside, a major active component of Renshen, has strong pharmacological activities, such as immune function stimulation, cardiovascular system protection, antitumor proliferation, antitumor invasion, and antitumor metastasis[13]. Shengjiang, with a long clinical history, has various biological activities, including anti-inflammatory, antimicrobial, and antiemetic activities[14]. Modern preclinical studies have also shown that Shengjiang is effective in ameliorating the side effects of doxorubicin and cisplatin and exerts chemosensitization effects in certain tumor cells[15].

The relationships among drugs, active components, and diseases can be studied using the network pharmacology of traditional Chinese medicine, which conforms to the characteristics of traditional Chinese medicine with multiple components, targets, and pathways[16,17]. They can overcome the difficulty of elucidating the mechanism of action of prescriptions against diseases because prescriptions used in traditional Chinese medicine contain a variety of medicinal materials with complex chemical compositions. Traditional Chinese medicine network pharmacology provides a novel strategy for developing traditional Chinese medicine and elucidating its underlying mechanisms. This study utilized network pharmacology to explore the mechanism of Wuzhuyu decoction against HCC and reveal active compounds, key genes, and potential pathways of Wuzhuyu decoction treatment of HCC, which would be useful for further investigation (Figure 1).

## MATERIALS AND METHODS

### Screening potential active compounds and related targets of Wuzhuyu decoction

The relationships among drugs, targets, and diseases were determined using the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, version 2.3, <https://tcmsp-e.com/>), a systematic pharmacological platform for Chinese herbal medicines[18]. All herbal compounds used in Wuzhuyu decoction were retrieved by searching “Wuzhuyu,” “Renshen,” “Shengjiang,” and “Dazhao” as the keywords. Based on the absorption, distribution, metabolism, and excretion protocols, the active compounds were screened out with oral bioavailability  $\geq 30$  and drug-likeness  $\geq 0.18$  as the criteria[19]. Potential target proteins of selected active compounds were identified in TCMSP. Each of the potential target proteins were then used as keywords searched using the UniProt database (<https://www.uniprot.org/>)[20] with the “*Homo sapiens*” species. Finally, the potential target genes of Wuzhuyu decoction and the corresponding UniProt ID of the potential targets were obtained.

### Screening targets relevant to HCC

The GeneCards (<https://www.genecards.org/>)[21], Online Mendelian Inheritance in Man (<https://omim.org/>)[22], MalaCards (<https://www.malacards.org/>)[23], DisGeNET (<https://www.disgenet.org/>)[24], Therapeutic Target Database (<http://db.idrblab.net/ttd/>)[25], and Comparative Toxicogenomics Database (CTD) (<http://ctdbase.org/>)[26] were searched using the keyword “hepatocellular carcinoma” to identify targets relevant to HCC. In the search results of GeneCards, the targets with relevance score  $\geq 10$  were screened. In the search results of DisGeNET, the targets with score  $\geq 0.1$  were screened. In the search results of CTD, the targets with inference score  $\geq 50$  were screened. After integrating and removing duplicates, a set of potential targets relevant to HCC were obtained and screened from the six databases mentioned above.

### Targets analysis and network construction

According to the potential target genes of Wuzhuyu decoction and targets relevant to HCC, a Venn online tool (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) was used to identify common targets that were potential target genes of Wuzhuyu decoction against HCC. Cytoscape software (version 3.8.2)[27] was used to construct the interaction between Wuzhuyu decoction-active compounds and target genes.

### Protein-protein interaction analysis

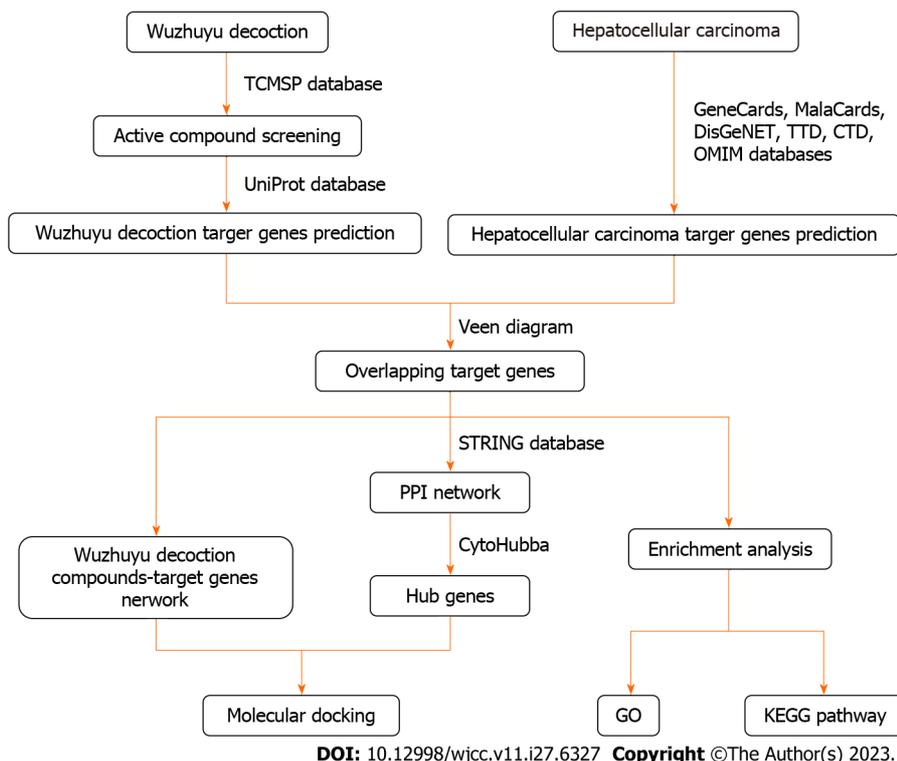
The STRING database (<http://string-db.org/>; version 11.0) is a database of known and predicted protein-protein interactions (PPIs). The PPI analysis was obtained from this database with the species limited to “*Homo sapiens*” and confidence score  $> 0.4$ [28,29]. The PPI analysis data were then imported into the Cytoscape software (version 3.8.2) to construct the network. Subsequently, the first 10 nodes, as hub genes, were identified *via* the following plug-in 12 CytoHubba algorithms of the Cytoscape software: betweenness; bottleneck; closeness; clustering coefficient; degree; density of maximum neighborhood component; EcCentricity; Edge Percolated Component; Maximal Clique Centrality; Maximum Neighborhood Component; radiality; and stress[30]. A Sankey diagram (<https://www.omicstudio.cn/tool/>) was constructed according to the hub genes, active compounds, and herbs of Wuzhuyu decoction.

### Enrichment analysis

The Gene Ontology (GO)[31] and Kyoto Encyclopedia of Genes and Genomes (KEGG)[32] pathway enrichment analyses were performed by the clusterProfiler package in R (R 4.1.0 for Windows) to confirm the biological processes (BPs) and signaling pathway of Wuzhuyu decoction active compounds involved in the treatment of HCC. An adjusted *P* value of  $< 0.05$  was considered statistically significant, which validated the enriched terms.

### Molecular docking

AutoDock Vina[33] was used to conduct molecular docking among the top 10 hub genes screened using the plug-in CytoHubba of the Cytoscape software and the key active components obtained in the Sankey diagram. The SDF format of ligands and PDB format of proteins were downloaded from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and RCSB PDB databases (<https://www.rcsb.org/>). The ligands were then imported into ChemBio3D Ultra (ChemBio3D Ultra 14.0.0.117 for Windows) to transfer the format to MOL2. Proteins were dehydrated and separated using the PyMol software (<https://pymol.org/2/>; version 2.5). Ligands and proteins were then introduced into AutoDock Tools for molecular docking. Vina scores between hub genes and key active components were obtained. When the Vina score was



**Figure 1** Schematic diagram of this work to explore potential mechanisms of Wuzhuyu decoction treating hepatocellular carcinoma. CTD: Comparative Toxicogenomics Database; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; OMIM: Online Mendelian Inheritance in Man; PPI: Protein-protein interaction; TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; TTD: Therapeutic Target Database.

lower than 0, molecular docking was considered successful. A lower Vina score indicated a higher binding affinity between the protein and the ligand. PyMol software was used to visualize the complexes of proteins and ligands.

## RESULTS

### Screening potential active compounds of Wuzhuyu decoction

The TCMSP database was used to screen for potential active compounds contained in Wuzhuyu decoction. In the TCMSP database, 764 compounds were identified, including 176 from Wuzhuyu, 190 from Renshen, 265 from Shengjiang, and 133 from Dazhao. Based on the standard of oral bioavailability  $\geq 30$  and drug-likeness  $\geq 0.18$ , there were 86 active compounds of Wuzhuyu decoction that were searched, including 30 from Wuzhuyu, 22 from Renshen, 5 from Shengjiang, and 29 from Dazhao. After removing duplicates, there were 77 active compounds. They were used for subsequent analysis. [Supplementary Table 1](#) shows essential information on some active compounds in the Wuzhuyu decoction.

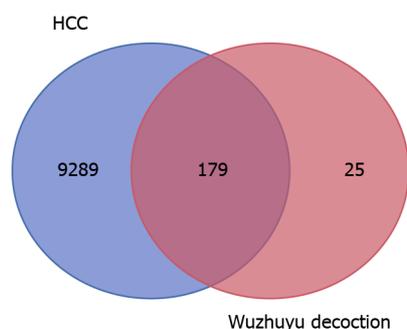
### Screening putative target genes of Wuzhuyu decoction

Using the retrieval and screening function of the TCMSP database, it was confirmed that 56 of the 77 active compounds in Wuzhuyu decoction have target proteins, and 187 proteins were targeted. Subsequently, the UniProt database was then utilized to transform the target proteins predicted by the active compounds of the Wuzhuyu decoction into the corresponding gene names. In total, 204 possible target genes were identified.

### Screening the potential therapeutic targets of Wuzhuyu decoction against HCC

A total of 9468 potential therapeutic target genes for HCC were identified after searching the GeneCard, Online Mendelian Inheritance in Man, MalaCard, DisGeNET, Therapeutic Target Database, and CTD databases. Based on the 204 possible target genes of the active compounds of Wuzhuyu decoction and potential therapeutic target genes related to HCC, the Venn online platform was used to draw a Venn diagram. As shown in [Figure 2](#), 197 core target were obtained, which were potential target genes of Wuzhuyu decoction against HCC. [Supplementary Table 2](#) shows the basic information of the 179 potential target genes of Wuzhuyu decoction against HCC.

Traditional Chinese medicine prescriptions with multiple targets have shown various pharmacological activities. Studying the possible mechanisms of traditional Chinese medicine prescriptions is highly significant in the treatment of diseases through network analysis. [Figure 3](#) shows the Wuzhuyu decoction active compounds and target genes network of Wuzhuyu decoction treating in HCC, which was structured using Cytoscape software. It can visually show the association between active compounds and target genes.



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**Figure 2** Venn diagram of Wuzhuyu decoction related targets and hepatocellular carcinoma related targets. HCC: Hepatocellular carcinoma.

### Constructing the PPI network and screening the hub genes of Wuzhuyu decoction against HCC

The PPI network can be used to identify potential targets of Wuzhuyu decoction in HCC treatment. Based on the 179 potential target genes identified, the PPI network was obtained using the STRING database, which involved 178 nodes and 1040 edges. The obtained data were further visualized using Cytoscape software (Figure 4). According to the 12 CytoHubba algorithms (see "PPI analysis") of Cytoscape software, the top 10 hub genes were selected based on each algorithm. According to the results, the top 10 hub genes of the 12 algorithms contained 44 different genes. In addition, the chord diagram (Figure 5) showed the relationship between the 44 genes and the corresponding algorithms. Based on the number of algorithms corresponding to genes, those 44 genes were sorted, and the top 10 hub genes were identified as tumor necrosis factor (TNF), interleukin (IL) 6, AKT1, TP53, caspase-3 (CASP3), mitogen-activated protein kinase 1 (MAPK1), epidermal growth factor receptor (EGFR), MYC, MAPK8, and JUN. These results were close to closeness, degree, Maximum Neighborhood Component, and radiality algorithms, which contain the same genes and are only slightly different in sequence, and are the closest to the radiality result (Figure 6).

Additionally, a Sankey diagram (Figure 7) was constructed based on the top 10 hub genes, active components, and traditional Chinese medicines of the Wuzhuyu decoction, in which MOL000098 (quercetin) targeted most of the top 10 hub genes. MOL000422 (kaempferol), MOL005344 (ginsenoside Rh2), MOL002662 (rutaecarpine), MOL002773 ( $\beta$ -carotene), and MOL000358 ( $\beta$ -sitosterol) were the main components of the Wuzhuyu decoction against HCC.

### GO and KEGG pathway enrichment analysis

GO and KEGG pathway enrichment analyses were conducted in clusterProfiler of R to elucidate the biological mechanism of Wuzhuyu decoction against HCC. The 179 potential targets were entered into R, and 2513 GO terms were obtained (adjusted,  $P < 0.05$ ), including 2257 BP, 199 molecular function (MF), and 57 cellular component terms. The top 10 of GO-BP terms, GO-molecular function terms, and GO-cellular component terms (Table 1) were sorted according to the adjusted  $P$  values from small to large. The top 10 GO enrichment items were selected for further visualization using bar (Figure 8A) and bubble diagrams (Figure 8B).

The 179 potential targets of Wuzhuyu decoction against HCC were put into R, and 177 enriched KEGG pathways were obtained (adjusted,  $P < 0.05$ ). Based on the adjusted  $P$  values ranging from small to large, the top 20 KEGG pathway enrichment terms were selected and further visualized using a bar diagram (Figure 9). "Hepatocellular carcinoma," as the keyword, was searched in the KEGG database to obtain potential pathways related to HCC. From the 177 results of enriched KEGG pathways, 64 were selected, which were possibly associated with HCC. They included the MAPK, p53, phosphatidylinositol 3-kinase (PI3K)-Akt, Janus kinase (JAK)-signal transducer of activators of transcription (STAT), and Hippo signaling pathways, among others. Details of the 64 pathways can be found in Supplementary Table 3. Finally, a network of target genes and pathways was constructed using the Cytoscape software (Figure 10).

### Molecular docking results

Based on the CytoHubba algorithm analysis results of the Cytoscape software, the top 10 hub genes were *TNF*, *IL6*, *AKT1*, *TP53*, *CASP3*, *MAPK1*, *EGFR*, *MYC*, *MAPK8*, and *JUN*, which were core target proteins. According to the Sankey diagram (Figure 7), the key active components were MOL000098 (quercetin), MOL000422 (kaempferol), MOL005344 (ginsenoside Rh2), MOL002662 (rutaecarpine), MOL002773 ( $\beta$ -carotene), and MOL000358 ( $\beta$ -sitosterol). Based on these results, molecular docking of the core target proteins and active compounds was performed.

The 10 core target proteins were molecularly docked with the six key active components using AutoDock Vine. A heat map was used to visualize the results of the docking score with the highest affinity, as shown in Figure 11. The binding energies between core target proteins and the key active components were between -5.9 and -10.4 kcal/mol, which indicated that the six key active components of Wuzhuyu decoction all bound well with the 10 core target proteins. In addition, TNF had a high affinity for all key active components. Finally, the top three core target proteins and key active component molecules with the best docking affinities were selected for visualization using PyMOL (Figure 12).

**Table 1 Top 10 Gene Ontology enrichment items**

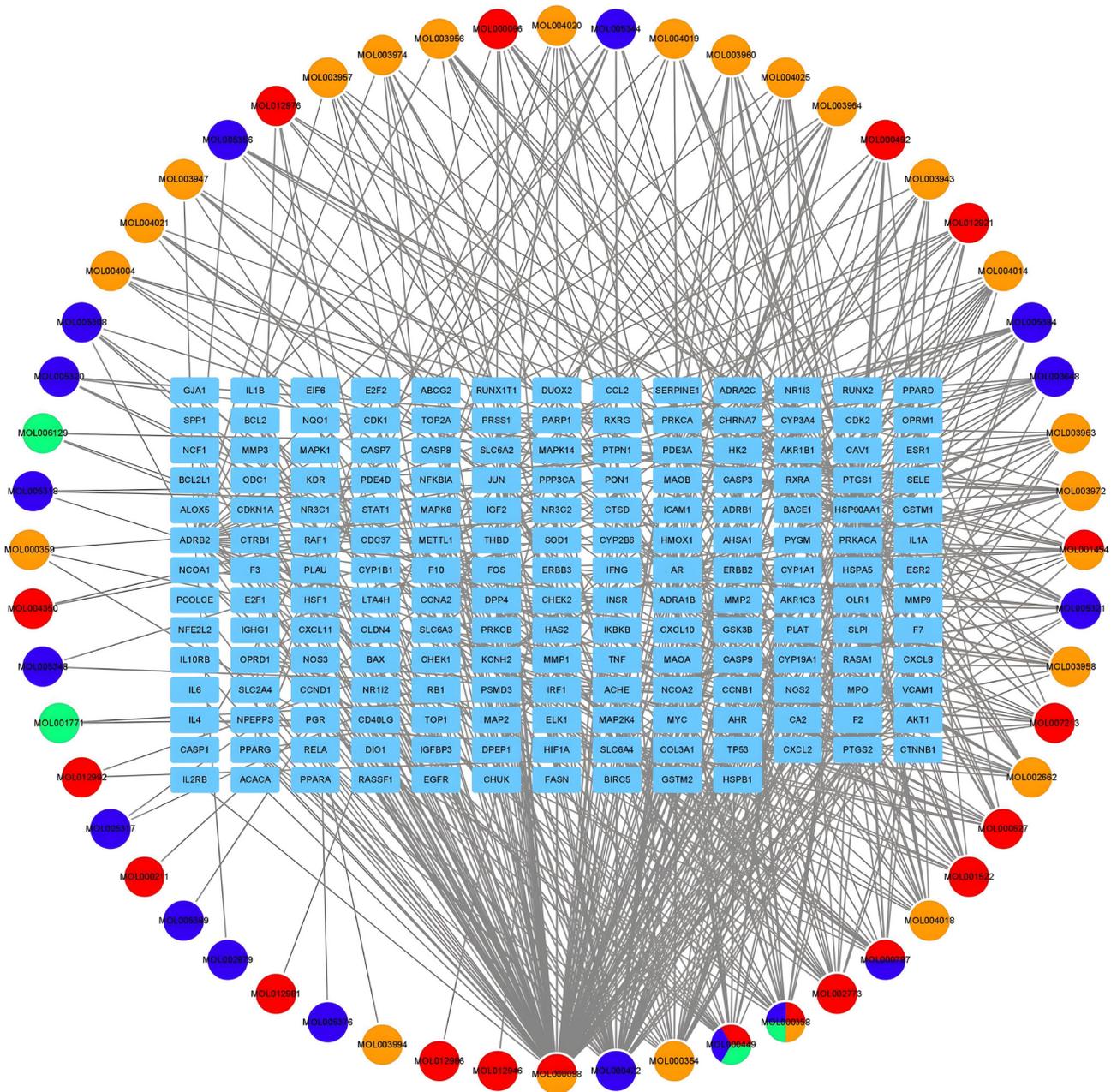
ID	Description	P value	Adjust P value	Gene number	GO items
GO:0032496	Response to lipopolysaccharide	2.70E-29	1.11E-25	37	BP
GO:0042493	Response to drug	5.99E-29	1.23E-25	38	BP
GO:0002237	Response to molecule of bacterial origin	2.40E-28	3.27E-25	37	BP
GO:0062197	Cellular response to chemical stress	4.08E-27	4.17E-24	36	BP
GO:0072593	Reactive oxygen species metabolic process	1.25E-26	1.02E-23	33	BP
GO:0010038	Response to metal ion	1.37E-24	9.36E-22	34	BP
GO:0070482	Response to oxygen levels	2.05E-24	1.20E-21	35	BP
GO:0001666	Response to hypoxia	1.28E-23	6.56E-21	33	BP
GO:0000302	Response to reactive oxygen species	1.99E-23	9.03E-21	28	BP
GO:0034599	Cellular response to oxidative stress	2.21E-23	9.04E-21	31	BP
GO:0045121	Membrane raft	1.49E-20	2.36E-18	29	CC
GO:0098857	Membrane microdomain	1.49E-20	2.36E-18	29	CC
GO:0044853	Plasma membrane raft	6.06E-09	6.38E-07	11	CC
GO:1902911	Protein kinase complex	4.05E-08	3.20E-06	10	CC
GO:0000307	Cyclin-dependent protein kinase holoenzyme complex	1.18E-07	6.68E-06	7	CC
GO:1902554	Serine/threonine protein kinase complex	1.27E-07	6.68E-06	9	CC
GO:0090575	RNA polymerase II transcription regulator complex	4.95E-07	2.24E-05	11	CC
GO:0005901	Caveola	7.06E-07	2.79E-05	8	CC
GO:0005667	Transcription regulator complex	5.82E-06	0.000192173	15	CC
GO:0031968	Organelle outer membrane	6.14E-06	0.000192173	11	CC
GO:0140297	DNA-binding transcription factor binding	5.06E-17	9.61E-15	28	MF
GO:0004879	Nuclear receptor activity	5.46E-17	9.61E-15	14	MF
GO:0098531	Ligand-activated transcription factor activity	5.46E-17	9.61E-15	14	MF
GO:0061629	RNA polymerase II-specific DNA-binding transcription factor binding	2.32E-15	3.06E-13	23	MF
GO:0003707	Steroid hormone receptor activity	1.78E-12	1.88E-10	9	MF
GO:0044389	Ubiquitin-like protein ligase binding	2.35E-10	2.07E-08	19	MF
GO:0001223	Transcription coactivator binding	4.22E-09	2.70E-07	7	MF
GO:0031625	Ubiquitin protein ligase binding	4.53E-09	2.70E-07	17	MF
GO:0017171	Serine hydrolase activity	4.71E-09	2.70E-07	14	MF
GO:0051879	Hsp90 protein binding	5.12E-09	2.70E-07	8	MF

BP: Biological process; CC: Cellular component; GO: Gene Ontology; Hsp90: Heat shock protein 90; MF: Molecular function.

## DISCUSSION

HCC is one of the most common cancers worldwide. As medical treatment and care continue to improve, morbidity and mortality rates for most tumors are declining. However, the mortality rate of HCC continues to increase[34]. The majority of the patients diagnosed with HCC progress to advanced stages of the disease quite soon and become ineligible for curative treatments. However, recurrence rates 5 years after liver resection have been reported to exceed 70%[34]. Presently, drug therapy has become an important method to prolong the overall survival and improve the quality of life of patients.

Traditional Chinese medicine has a long history and is widely used for treating various diseases. However, the choice of adjuvant therapies for HCC remains limited. In the guidelines of the Chinese Society of Clinical Oncology, modern traditional Chinese medicine preparations (Huai Er granules, elemene, compound cantharidin capsule, *etc*) and traditional Chinese medicine syndrome differentiation treatments have been included in the guidelines and are recommended as level I treatments. Traditional Chinese medicine prescriptions comprise multiple herbs that can act on



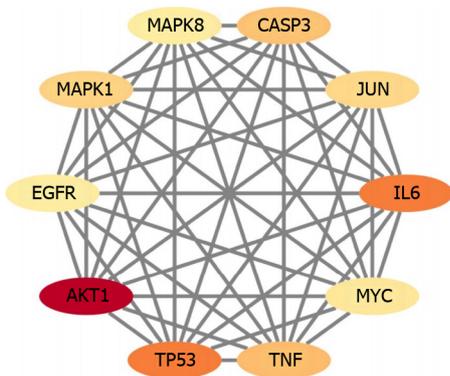
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**Figure 3 “Wuzhuyu decoction-active compounds-target genes” network.** The blue rectangles represent the potential targets. The ellipses represents the components contained in Wuzhuyu decoction. The red represents Dazao, the orange represents Wuzhuyu, the green represents Shengjiang, and the purple represents Renshen.

multiple targets and pathways simultaneously and have a wide range of pharmacological activities. The occurrence and development of HCC are complex processes involving multiple etiologies, genes, and steps, including gene mutations, changes in protein expression, and pathway activation. Traditional Chinese medicine may be helpful in HCC treatment. Studies have revealed that when used as an adjuvant therapy it may not only prolong the overall survival time of HCC patients but also enhance the anti-HCC efficacy of sorafenib by promoting tumor apoptosis and regulating inflammation in the tumor microenvironment[35,36]. Traditional Chinese medicine is characterized by several active ingredients, targets, and complex pathways of action, which make the mechanism of its treatment unclear and difficult to study.

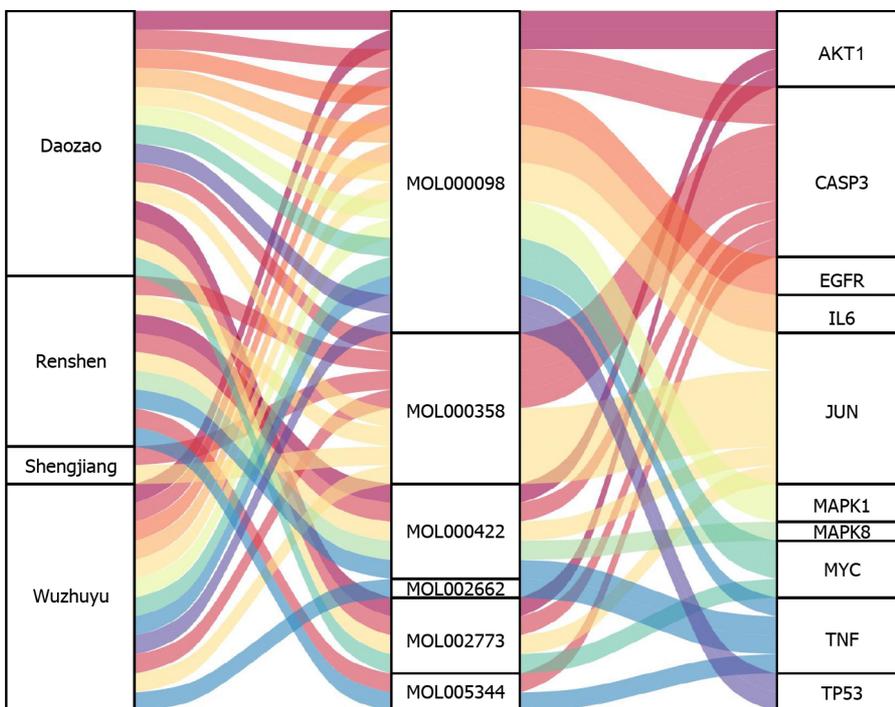
Network pharmacology emphasizes a holistic approach to treatment. It analyzes the relationship between drugs and diseases by predicting potential targets, constructing protein networks, and performing molecular target docking, which is a research concept consistent with the holism of traditional Chinese medicine. Network pharmacology has been widely used to explain the mechanisms underlying Chinese medicine. The specific distribution of the active ingredients in the body is unclear. The results of network pharmacological analysis are predicted based on the network database. The sources of information, theory, and experimental results for each database may be inconsistent, and the analysis results must be verified experimentally.





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**Figure 6 Top 10 hub genes were confirmed by radiality.** The darker the color of the node, the better the degree. *CASP3*: Caspase-3; *EGFR*: Epidermal growth factor receptor; *IL6*: Interleukin 6; *MAPK*: Mitogen-activated protein kinase; *TNF*: Tumor necrosis factor.



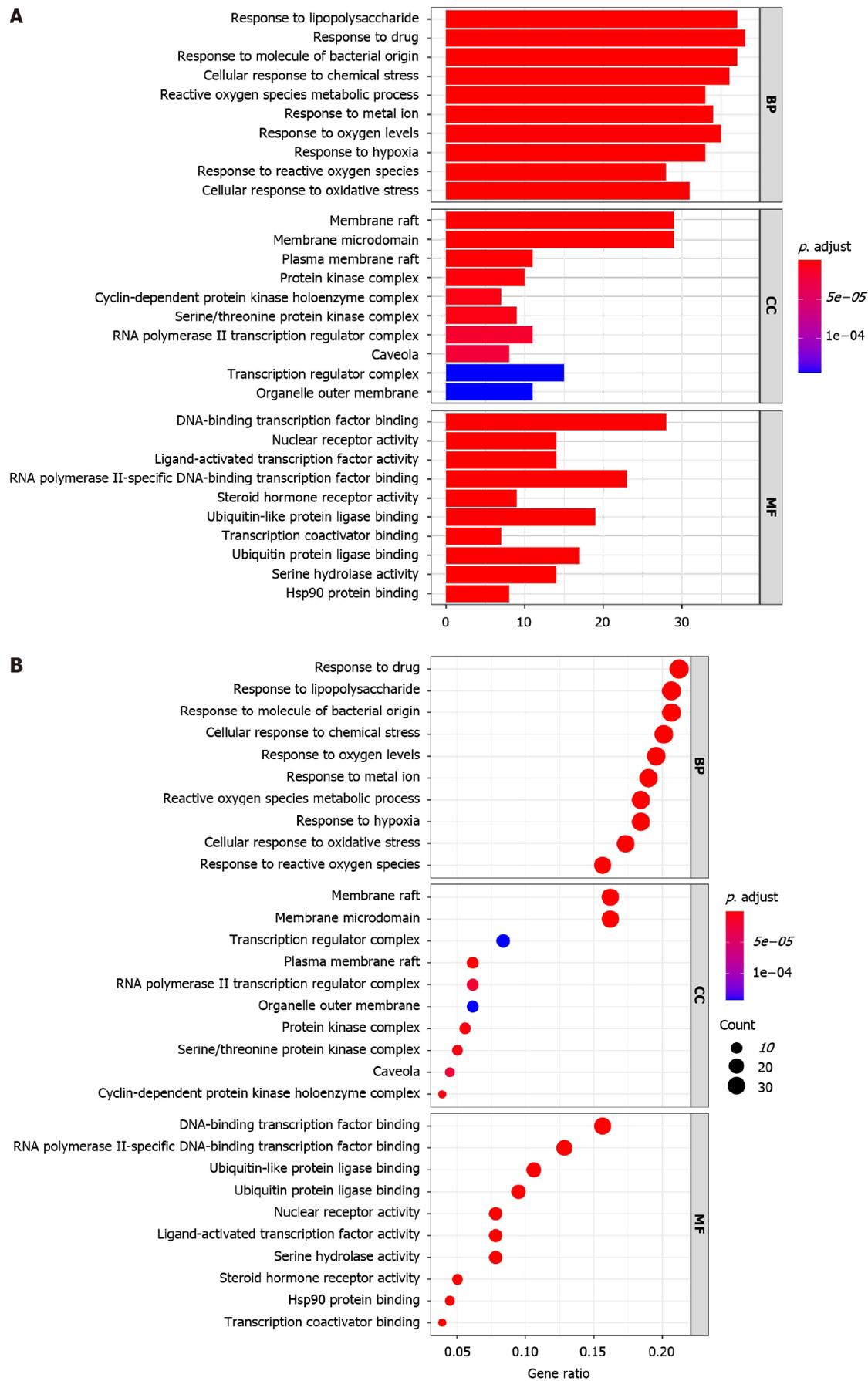
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**Figure 7 Sankey diagram showed the relationship among the target genes, the active components, and traditional Chinese medicines of Wuzhuyu decoction.** The vertical bars on the left represent the different traditional Chinese medicines, the middle bars represent the active components, and the right bars represent the target genes. The size of the bars and the thickness of the lines represent the amount of interactions. *CASP3*: Caspase-3; *EGFR*: Epidermal growth factor receptor; *IL6*: Interleukin 6; *MAPK*: Mitogen-activated protein kinase; MOL000098: Quercetin; MOL000422: Kaempferol; MOL005344: Ginsenoside Rh2; MOL002662: Rutaecarpine; MOL002773:  $\beta$ -carotene; MOL000358:  $\beta$ -sitosterol; *TNF*: Tumor necrosis factor.

Nevertheless, network pharmacology breaks the “one disease-one target-one drug” approach of clinicians. It focuses on understanding diseases from the perspective of systems and biological networks, analyzing the molecular association between drugs and their therapeutic targets and revealing the systemic pharmacological mechanisms of drugs. This can guide new drug development and clinical treatment. Network pharmacology provides a new method for scientifically interpreting the principles of traditional Chinese medicine and solves problems through well-informed development.

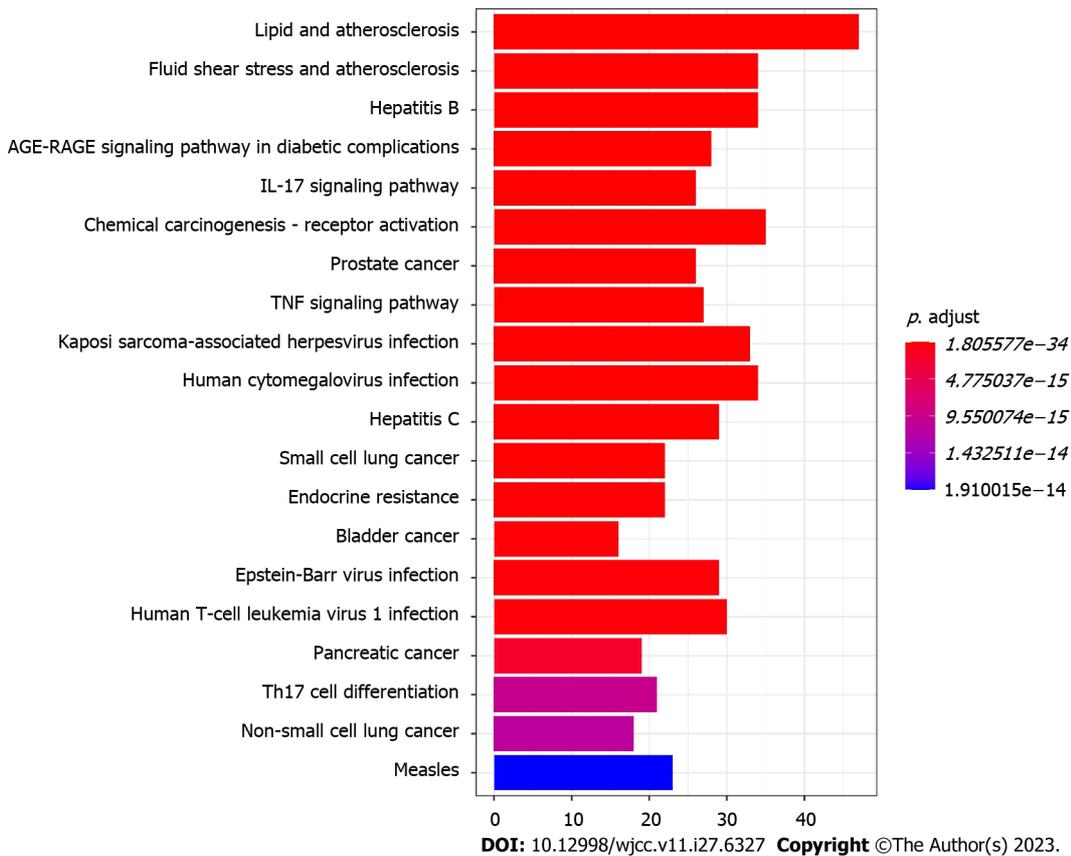
In the past 3 years, several studies have analyzed the mechanism of action of traditional Chinese medicine through network pharmacology and verified its accuracy through experiments. Network pharmacology technology can elucidate the chemical composition, action targets, pharmacological mechanisms, and formulation laws of traditional Chinese medicine prescriptions, and the recognition and acceptance of traditional Chinese medicine will continue to improve. This study adopted network pharmacology to preliminarily reveal the potential therapeutic mechanism of Wuzhuyu decoction in HCC.

Wuzhuyu decoction has been used to treat digestive diseases. Studies have shown that alkaloids extracted from *Evodia* have anti-liver cancer activity *in vitro* and *in vivo* through targets such as NOD1, AKT, and WWOX[37-39]. This study



**Figure 8 Diagram of the top 10 Gene Ontology enrichment items (biological process, cellular component, molecular function).** A: Bar diagram of the top 10 Gene Ontology enrichment items; B: Bubble diagram of the top 10 Gene Ontology enrichment items. BP: Biological process; CC: Cellular

component; MF: Molecular function; Hsp90: Heat shock protein 90.



**Figure 9** Bar diagram of the top 20 Kyoto Encyclopedia of Genes and Genomes enrichment items. IL6: Interleukin 6; Th17: T helper type 17; TNF: Tumor necrosis factor.

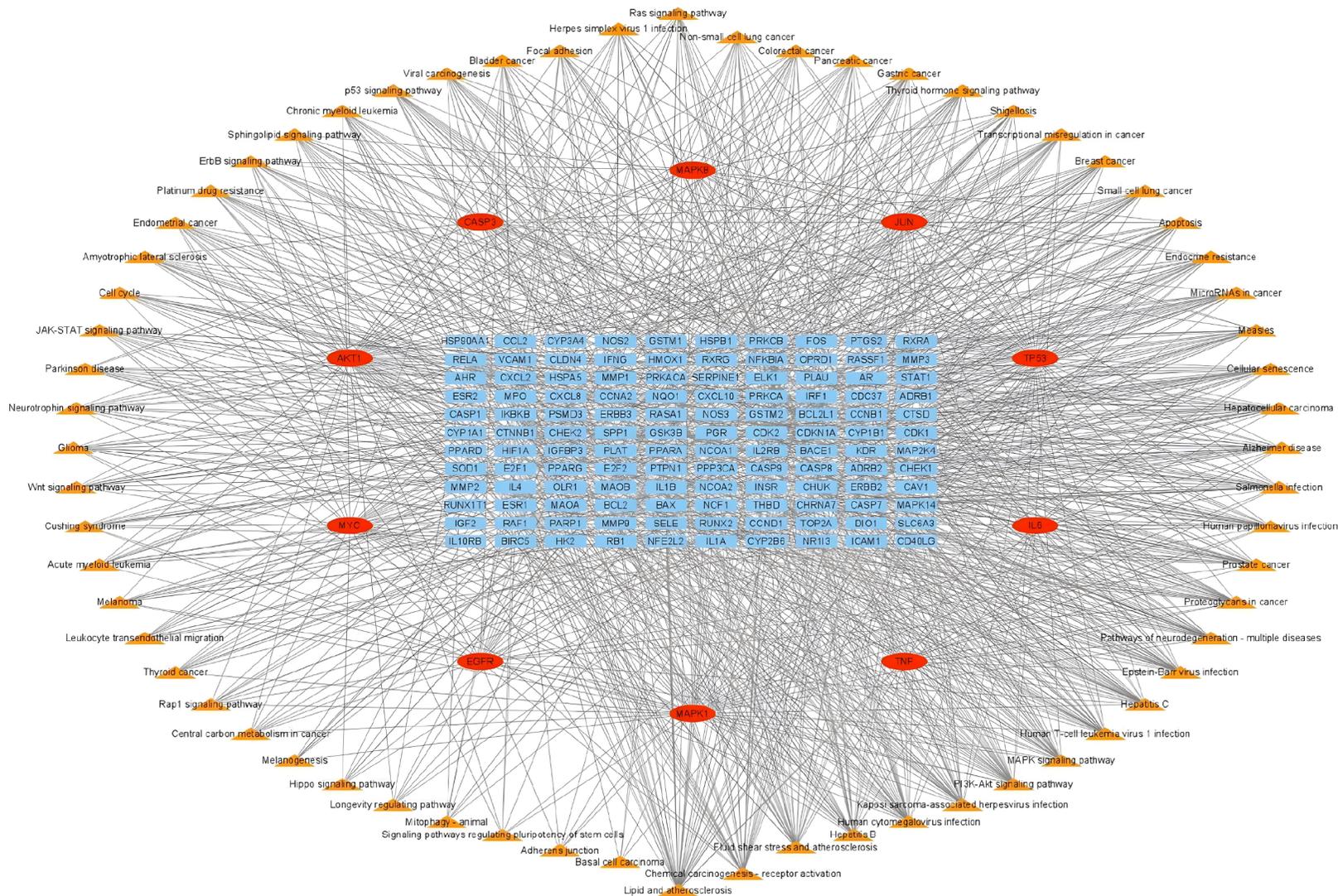
analyzed the active components of Wuzhuyu decoction and constructed an interaction network diagram of active compounds and target genes. It was observed that the six main active components (quercetin, kaempferol, ginsenoside Rh2, rutaecarpine,  $\beta$ -carotene, and  $\beta$ -sitosterol) may be the main components of Wuzhuyu decoction in adjuvant therapy of HCC.

Quercetin is a flavonoid with antioxidant, anti-inflammatory, and antitumor activities. It has notable antiproliferative and antiapoptotic effects in treating HCC and has a regulatory effect on the cell cycle of tumors[40]. Furthermore, quercetin has been shown to not only regulate apoptosis, migration, and invasion of cells by blocking the JAK2/STAT3 signaling pathway but also inhibit glycolysis by reducing hexokinase 2 and inhibiting the AKT/mammalian target of rapamycin signaling pathway, ultimately hindering the progression of HCC[41,42].

Kaempferol, also a member of the flavonoids, can inhibit both MAPK and hypoxia-inducible factor-1 activity at physiologically relevant concentrations (5-10 mM), reduce liver fibrosis by targeted binding to the ATP-binding pocket of ALK5, and effectively suppress the survival of HCC cells under hypoxia[43,44]. In addition, a natural compound extracted from *Abies georgei*, which is structurally related to kaempferol, has been shown to enhance the tumor immunosuppressive microenvironment and strengthen the therapeutic effects of low-dose sorafenib without obvious toxicity[45]. It also enhanced the efficacy of sorafenib in HCC *in vitro*[46].

Recently, ginsenosides have been shown to affect a variety of tumors. Ginsenoside Rh2 has been shown to decrease EZH2, downregulate *p14*, *p15*, and *p16* mRNA levels, inhibit HCC cell proliferation[47], and inhibit HCC cell growth,  $\beta$ -catenin, and autophagy[48].

$\beta$ -sitosterol is a phytosterol with antioxidant activity that has ameliorative effects on HCC, and its structural optimization can improve the efficacy of HCC treatment[49]. Rutaecarpine alleviates weight loss, liver injury, and inflammation[50]. Long-term  $\beta$ -carotene treatment can increase blood natural killer, IL2, TNF- $\alpha$ , white blood cells, total protein, albumin, and A/G levels and decrease blood alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities in HCC in rats[51]. Although rutaecarpine and  $\beta$ -carotene have no direct antitumor activity, they can slow the development of tumors, which is beneficial for inhibiting the progression of HCC. These results support the predicted results of network pharmacology. This also proves the feasibility of network pharmacology in identifying and analyzing the mechanisms of action of traditional Chinese medicine. This study preliminarily revealed the potential active substance of Wuzhuyu decoction in treating HCC.



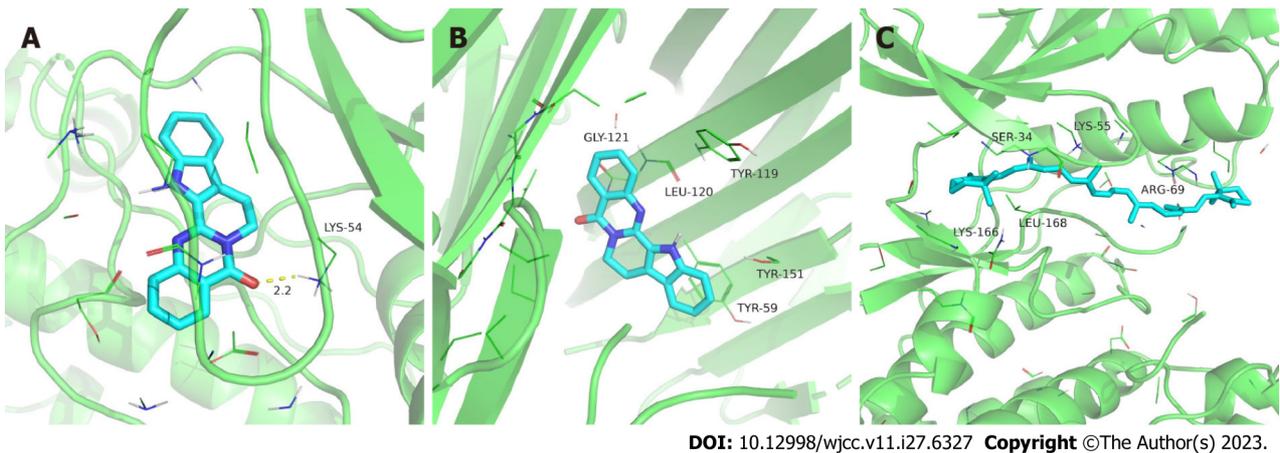
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**Figure 10** “Target genes-pathways” network. The blue rectangles represent the potential targets of Wuzhuyu decoction against hepatocellular carcinoma (HCC). The red ellipses represent the top ten hub genes of Wuzhuyu decoction against HCC; the brown triangles represent potential pathways related to HCC. *CASP3*: Caspase-3; *EGFR*: Epidermal growth factor receptor; *IL6*: Interleukin 6; *MAPK*: Mitogen-activated protein kinase; *TNF*: Tumor necrosis factor.

-8.6	-8.3	-9.2	-8.1	-8.8	-9.7	TNF
-7.3	-7.2	-7.3	-6.9	-7.0	-8.0	IL6
-6.8	-5.9	-6.7	-6.2	-6.4	-7.3	AKT1
-7.8	-7.2	-8.7	-7.8	-7.7	-9.1	TP56
-7.1	-6.3	-7.8	-8.1	-8.0	-8.3	CASP3
-9.3	-6.3	-8.0	-9.0	-9.1	-10.4	MAPK1
-8.5	-7.8	-9.0	-8.0	-8.2	-9.3	EGFR
-6.9	-6.5	-7.3	-6.2	-6.3	-7.4	MYC
-10.1	-7.7	-7.6	-8.1	-8.6	-8.8	MAPK8
-6.6	-6.0	-7.9	-6.3	-6.4	-7.8	JUN
Beta-carotene	Beta-sitosterol	Ginsenoside rh2	Kaempferol	Quercetin	Rutacarpine	

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**Figure 11 Heat map of the docking scores of the key active components and the core target proteins of Wuzhuyu decoction.** CASP3: Caspase-3; EGFR: Epidermal growth factor receptor; IL6: Interleukin 6; MAPK: Mitogen-activated protein kinase; TNF: Tumor necrosis factor.



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**Figure 12 Molecular docking results.** A: Rutacarpine to mitogen-activated protein kinase 1; B: Rutacarpine to tumor necrosis factor; C:  $\beta$ -carotene to mitogen-activated protein kinase 8.

According to the results of comprehensive drug target prediction and pathway analysis, Wuzhuyu decoction may exert an antitumor effect on HCC by inhibiting cell proliferation and angiogenesis and by inducing cell apoptosis. The occurrence and development of HCC are accompanied by the dysregulation of multiple signaling pathways, including cell proliferation, invasion, migration, and angiogenesis. From a treatment perspective, dysregulated signaling pathways are also important targets for treating HCC.

In this study, network pharmacological methods were used to analyze and predict HCC, and the results showed that Wuzhuyu decoction had two main effects on HCC. First, it regulated HCC cell proliferation and apoptosis through the TP53, Ras-MAPK, PI3K-Akt, and JAK-STAT signaling pathways. TP53, a negative regulator of the cell cycle, is one of the most common protein variants expressed in cancer cells and plays important roles in cell cycle regulation, DNA damage repair, cell senescence, and apoptosis[52]. In addition, TP53 has been shown to influence the microenvironment and immune response, which can affect the rate of tumor expansion and aggressiveness[53]. The Ras-MAPK and PI3K-Akt signaling pathways are classical tumor signaling pathways that play crucial roles in regulating the proliferation, apoptosis, and metastasis of tumor cells. They are often considered key therapeutic targets for HCC, and many related small-molecule targeted inhibitors have entered clinical trials for HCC[54,55].

Second, Wuzhuyu decoction regulated HCC angiogenesis through the Hippo signaling pathway. Increasing evidence suggests that the Hippo signaling pathway plays a role in regulating vascular germination, vascular barrier formation, and vascular remodeling[56]. In addition, AKT has biological functions such as regulating cell survival, proliferation, and metabolism. Studies have revealed that AKT not only participates in the migration and invasion of cancer cells but also

promotes tumor angiogenesis and enhances vascular permeability[57]. Finally, the KEGG pathway analysis results also included signaling pathways related to lipids and atherosclerosis, hepatitis B, hepatitis C, and the AGE-RAGE signaling pathway in diabetic complications, which were related to the risk factors for HCC.

The results of this study indicate that Wuzhuyu decoction may inhibit HCC cell proliferation and angiogenesis by targeting P53, Ras-MAPK, PI3K-Akt, Hippo, and other signaling pathways *via* quercetin, kaempferol, ginsenoside Rh2, and other active compounds. However, network pharmacological analysis methods have some limitations. Regarding the detailed pharmacological mechanism of Wuzhuyu decoction in the treatment of HCC, we aim to further conduct accurate target verification and clinical effectiveness verification in subsequent studies.

## CONCLUSION

In this study, network pharmacology was used to integrate potential targets, construct networks, and perform enrichment analysis. It was preliminarily predicted that quercetin, kaempferol, ginsenoside Rh2, rutaecarpine,  $\beta$ -carotene, and  $\beta$ -sitosterol were identified as key components of Wuzhuyu decoction in the treatment of HCC. *TNF*, *IL6*, *AKT1*, *TP53*, *CASP3*, *MAPK1*, *EGFR*, *MYC*, *MAPK8*, and *JUN* may be important target genes in the pharmacological network. Furthermore, P53, Ras-MAPK, PI3K-Akt and Hippo signaling pathways may play an important role in the treatment of HCC by Wuzhuyu decoction. These results provide preliminary information and a basis for further exploration of the potential mechanism of action of Wuzhuyu decoction in HCC treatment.

## ARTICLE HIGHLIGHTS

### Research background

The occurrence and development of hepatocellular carcinoma (HCC) are complex processes involving multiple etiologies, genes, and steps, including gene mutations, changes in protein expression, and pathway activation. Traditional Chinese medicine prescriptions comprise multiple herbs that can act on multiple targets and pathways simultaneously and have a wide range of pharmacological activities. In the guidelines of the Chinese Society of Clinical Oncology, modern traditional Chinese medicine preparations and traditional Chinese medicine syndrome differentiation treatments have been included in the guidelines. Wuzhuyu decoction, as a warming interior formula, can warm the middle jiao (stomach, liver, spleen, and gallbladder). Recent studies have reported that it also exhibits anti-inflammatory and antitumor activities, induces cell cycle arrest, and suppresses tumor cell migration.

### Research motivation

This study utilized network pharmacology to explore the mechanism of Wuzhuyu decoction against HCC and reveal active compounds, key genes, and potential pathways of Wuzhuyu decoction treatment of HCC, which would be useful for further investigation.

### Research objectives

This study aimed to explore the potential mechanism of action of Wuzhuyu decoction against HCC.

### Research methods

This study utilized network pharmacology to explore the mechanism of Wuzhuyu decoction against HCC. Network pharmacology emphasizes a holistic approach to treatment. It analyzes the relationship between drugs and diseases by predicting potential targets, constructing protein networks, and performing molecular target docking, which is a research concept consistent with the holism of traditional Chinese medicine. Network pharmacology has been widely used to explain the mechanisms underlying Chinese medicine.

### Research results

This study analyzed the active components of Wuzhuyu decoction and constructed an interaction network diagram of active compounds and target genes. According to the analysis results, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were conducted. The results of this study indicate that quercetin, kaempferol, ginsenoside Rh2, and other active compounds in the Wuzhuyu decoction may be involved in the regulation of P53, Ras-MAPK, phosphatidylinositol 3-kinase (PI3K)-Akt, Hippo, and other signaling pathways by acting on the first 10 hub genes to achieve therapeutic effects on HCC.

### Research conclusions

In this study, network pharmacology was used to integrate potential targets, construct networks, and perform enrichment analysis. Wuzhuyu decoction may inhibit HCC cell proliferation and angiogenesis by targeting P53, Ras-MAPK, PI3K-Akt, Hippo, and other signaling pathways *via* quercetin, kaempferol, ginsenoside Rh2, and other active compounds.

### Research perspectives

Regarding the detailed pharmacological mechanism of Wuzhuyu decoction in the treatment of HCC, we will conduct

accurate target verification and clinical effectiveness verification in subsequent studies.

## FOOTNOTES

**Author contributions:** Ouyang JY, Lin WJ, and Dong JM primarily conducted this study; Yang Y and Yang HK provided technical support in network pharmacology and molecular docking; Zhou ZL and Wang RQ provided the concept, ideas support, and manuscript revision; All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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