

## Original Research Article

# A network pharmacology-based investigation on the underlying mechanism of Huqian Wan against osteoporosis

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### Abstract

**Purpose:** To investigate the underlying molecular mechanism of Huqian Wan (HQW) in the treatment of osteoporosis (OP) using a network pharmacology-based strategy.

**Methods:** The bioactive components and their targets of HQW, as well as OP-related targets were identified using public databases, and online predictive tools. Functional enrichment analyses were performed using Metascape platform. Network construction and analysis were conducted using Cytoscape software, while molecular docking was performed to analyze molecular binding affinities using AutoDock software.

**Results:** A database retrieval identification of HQW contained 128 active components and 482 targets, 85 of which overlapped with OP-related genes and were considered potential targets for HQW treatment of OP. Protein interaction analysis revealed five key targets, including AKT1, IL6, VEGFA, IL1B, and CASP3. Functional annotation and pathways enrichment analyses showed that the 85 targets were significantly enriched in interleukin-related signal pathways, inflammatory response, and cell proliferation. Moreover, network topology analysis showed that quercetin, kaempferol, nobiletin, tetradecanoic acid, and palmitic acid are the most important 5 active components. Sankey diagram illustrated the herb-component-target connections in osteoclast differentiation and demonstrated that PPARG was the most affected target. Molecular docking revealed the potential binding pose and affinity between PPARG and active compounds.

**Conclusion:** This systemic characterization of the active components and molecular mechanisms of HQW's treatment of OP will drive subsequent mechanistic and clinical research, and provide a new approach to understanding the mechanism of complex traditional Chinese medicine (TCM).

**Keywords:** Huqian Wan, Osteoporosis, Network pharmacology, Molecular docking, osteoclast

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## INTRODUCTION

Osteoporosis (OP), a systemic bone disorder, is characterized by reduced bone mass and bone

tissue destruction. It is commonly observed in elderly and postmenopausal women, leading to skeletal brittleness and raising the likelihood of fractures due to minor trauma or routine activities

[1,2]. It has now been acknowledged as a major threat to global health, with approximately 13 % of the Chinese population having been impacted [3]. It is generally believed that OP occurs as a result of abnormal bone remodeling mediated by osteoblasts and osteoclasts. In patients with rheumatoid arthritis, studies have revealed the contributions of increased inflammatory cytokines to OP [4]. Additionally, risk factors such as oxidative stress, glucocorticoid, bone immune dysfunction, and estrogen deficiency also contribute to the development of OP. Currently, many drug therapies are utilized to reduce OP symptoms including raloxifene, testosterone, and calcitonin [5]. However, these drugs for OP can be quite expensive and may not be as effective as desired, and may also cause side effects.

Traditional Chinese medicine (TCM) has been frequently employed in the prevention and therapy of OP because of its favorable efficacy and minor adverse effects [6]. *Huqian Wan* (HQW) is a well-known Chinese medicine used for strengthening muscles and bones and is particularly effective in treating osteoarthritis and OP. It is composed of 9 components, including BAISHAO (the root of *Cynanchum otophyllum* Schneid), CHENGPI (dried ripe peel of *Citrus reticulata* Blanco), ganjiang (dried root of *Zingiber officinale* Rosc.), gougou (bones of canine dog), guiban (plastron and carapace of *Chinemys reevesii*), huangbo (bark of *Phellodendron amurense* Rupr.), shu dihuang (*Rehmannia glutinosa* (Gaetn.) Libosch. ex Fisch. et Mey.), suoyang (*Cynomorium songaricum* Rupr.), and zhimu (*Anemarrhena asphodeloides* Bunge). *Huqian Wan* has been clinically used for 600 years, during which no major adverse effects have been reported. A recent study demonstrated that HQW improved weight-bearing asymmetry, decreased bone loss, and reduced the level of TNF- $\alpha$  and IL-1 $\beta$  in the affected joint in rats with anterior cruciate ligament transection (ACLT)-induced knee osteoarthritis (KOA) [7].

At present, the underlying mechanism of the anti-osteoporotic activities of HQW remains poorly understood. Given the complex nature of TCM – characterized by its multi-component, multi-target, and multi-pathway properties – it is challenging to elucidate the pharmacological mechanism of HQW using traditional methods. Fortunately, network pharmacology, as an analytical method of integrity, synergy, and dynamics, has been developed to solve the complicated interactions among herbs, compounds, and targets in the treatment of diseases. It has been adopted to elucidate the pharmacological mechanism of various TCMs on

OP at a holistic level [8-10]. In this study, the active components of HQW were retrieved from the TCMSP database and literature research. Then, the component targets and OP targets were collected and intersected to obtain the candidate targets. The underlying molecular mechanism of the action of HQW against OP was elucidated via enrichment analysis, network analysis, and molecular docking.

## METHODS

### Identification of active compounds and targets of HQW

The components and targets of HQW were obtained from Traditional Chinese Medicine Systems Pharmacology Database, and Analysis Platform (TCMSP, <https://lsp.nwu.edu.cn/tcmsp.php>). An assessment of the active components was performed using the following standards: Drug Similarity (DL)  $\geq 0.18$  and Oral Bioavailability (OB)  $\geq 30\%$ , and the corresponding targets were also obtained from TCMSP database. The components of GOUGU, GUIBAN, and SUOYANG were retrieved from published studies owing to the lack of relevant data in the TCMSP database, and their active components were identified according to the gastrointestinal absorption and drug-like parameters predicted using SwissADME online tool (<http://www.swissadme.ch/>). The targets of these active components were identified by merging the predicted results of Swiss Target Prediction (<http://www.swisstargetprediction.ch/>) and TargetNet (<http://targetnet.scbdd.com/>). Only human proteins were selected for further analysis.

### Identification of OP-related targets

The targets of OP were identified from Online Mendelian Inheritance in Man (OMIM) Database (<http://omim.org/>), Comparative Toxicogenomics Database (<http://ctdbase.org/>), and GeneCards Database (<https://www.genecards.org/>), and OP targets were identified via intersecting the genes from the above three databases. The overlapping genes between the OP targets and HQW targets were identified as candidate targets of HQW against OP.

### Functional enrichment analysis

Enrichment analyses were conducted using an online tool provided by the Metascape platform (<http://metascape.org/>). Briefly, the gene symbols of the candidate targets of HQW were submitted to Metascape platform, and then analyzed after

setting the *Homo sapiens* as organism reference. Protein-protein interaction (PPI) network was also generated in the Metascape platform. In addition, molecular complex detection (MCODE) algorithm was applied to identify and extract clusters with dense connections from the PPI network.

### Network analysis

The PPI information on the candidate targets was downloaded from the STRING (<https://string-db.org/>) database and imported into the Cytoscape 3.7.2 software to construct a PPI network. The herb-component (H-C) and herb-component-target-pathway (H-C-T-P) networks were also built. Topological analysis of the networks was performed via the Analyze Network tool in the Cytoscape software.

### Molecular docking

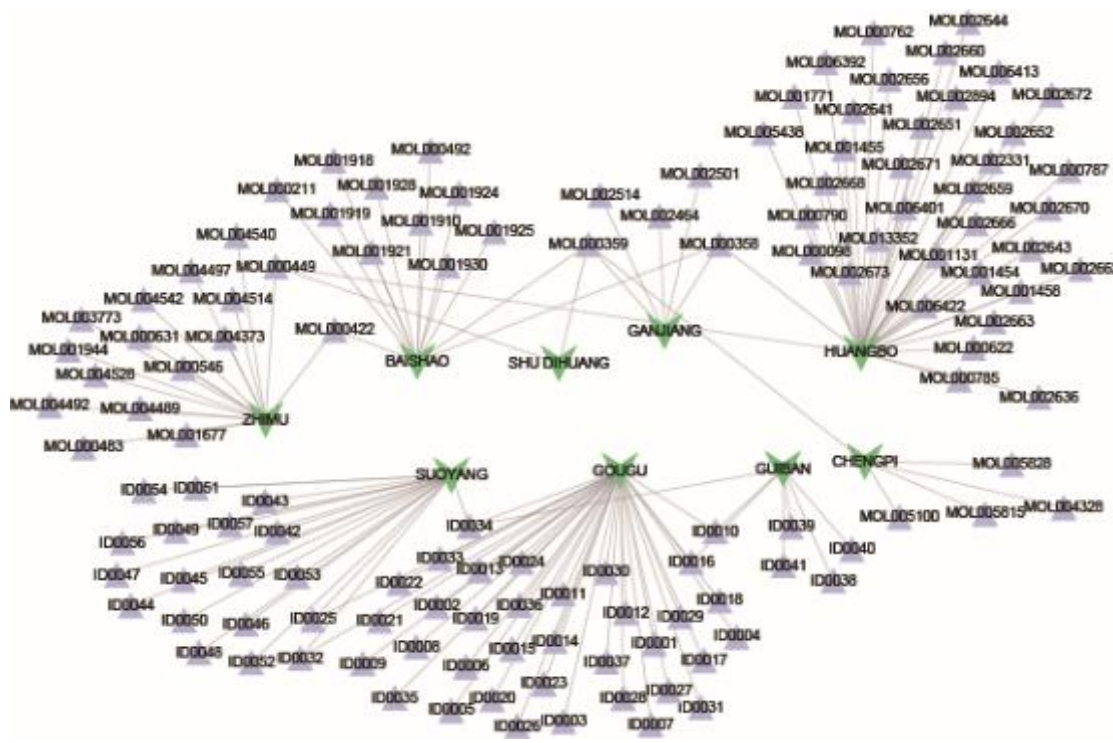
Sankey diagram (<http://sankeymatic.com/>) was generated with the genes related to osteoclast differentiation and active components of HQW to identify hub targets and components. The binding affinity between PPARG and its linked components was analyzed via molecular docking analysis. Firstly, the 3D structures of PPARG (PDB ID: 5ycp) were obtained from the RCSB PDB database (<https://www.rcsb.org/>), and downloaded the structure of the active compounds from PubChem database

(<https://pubchem.ncbi.nlm.nih.gov/>). The protein structure was prepared by removing water molecules, adding non-polar hydrxzogens, and computing the gasteiger charge. The grid box for docking was set to include the binding pocket for the binding of small-molecule ligands. Autodock Vina was used to dock the receptor protein with active components and calculate the binding affinities. The best conformations with the lowest binding energy were exported, and the 2D and 3D diagrams of the ligand-protein complex were generated using LigPlus and PyMOL, respectively.

## RESULTS

### Identification of the active components in HQW

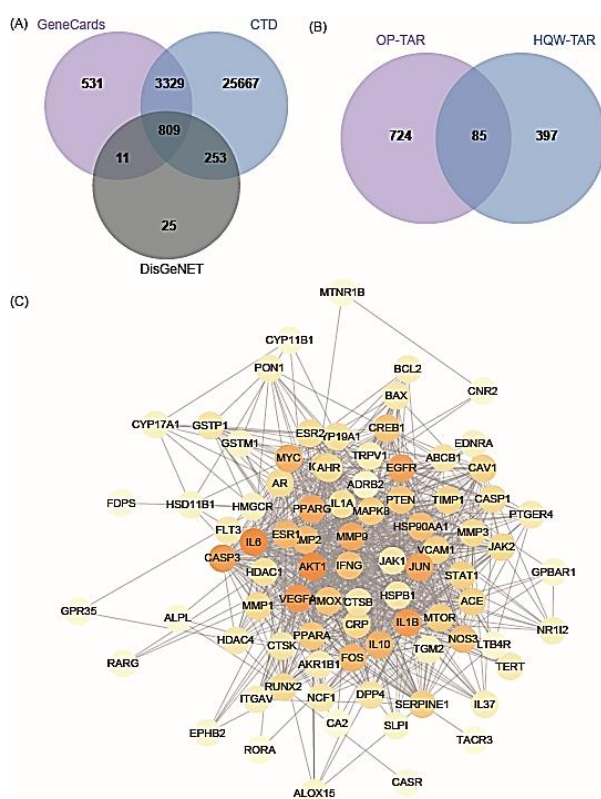
The active components of each herb in HQW were identified, and are illustrated in Figure 1. A total of 128 active compounds were identified in HQW, with 13 in BAISHAO, 5 in CHENGPI, 5 in GANJIANG, 37 in GOUGU, 7 in GUIBAN, 38 in HUANGBO, 2 in SHU DIHUANG, 19 in SUOYANG, and 16 in ZHIMU. Meanwhile, a total of 482 targets of these active compounds were identified. Specifically, there were 114 targets of ZHIMU, 206 of HUANGBO, 91 of BAISHAO, 31 of SHU DIHUANG, 68 of CHENGPI, 44 of GANJING, 83 of GUIBAN, 248 of GOUGU, and 146 of SUOYANG.



**Figure 1:** Herb-compound network of HQW. Green nodes represent herbs and purple triangles represent active ingredients

### Identification of OP-related targets and candidate targets of HQW against OP

The OP-related targets were identified via intersecting the results from the GeneCards, CTD, and DisGeNet database, and 809 intersected genes were considered as OP-related targets (Figure 2 A). A Venn diagram was constructed to further pinpoint potential therapeutic targets of HQW against OP, yielding 85 intersected targets that were related to both HQW and OP (Figure 2 B). The PPI information of the 85 candidate targets of HQW against OP was retrieved from the STRING database and used to construct a PPI network with 1009 interactions (Figure 2 C). Topological analysis revealed that IL6 and AKT were 2 targets with the highest degrees, suggesting that they may play a more pivotal role in the action of HQW against OP.

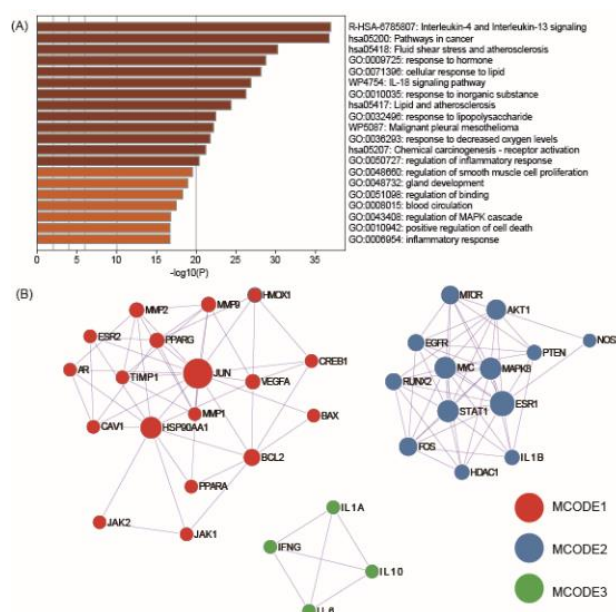


**Figure 2:** Identification of candidate targets of HQW in the treatment of OP. (A) Venn diagram shows the OP related-related genes in each database. (B) Identification of the candidate target by intersecting OP related-genes and HQW’s targets. (C) The PPI network of the 85 candidate targets of HQW against OP

### Enrichment analysis and MCODE analysis

To further elucidate the biological functions and signaling pathways involved in these potential genes, enrichment analysis was performed using

Metascape online tool. The results demonstrated that 4554 terms were enriched, including 241 canonical pathways, 1 category, 2 CORUM, 2942 GO biological processes, 450 KEGG pathways, 396 Reactome gene sets, and 622 wikipathways. The top 20 terms are illustrated in Figure 3 A. It was revealed that the candidate targets were significantly associated with interleukin signals, inflammatory response, cell proliferation, response to hormones, and inorganic substances. Through MCODE analysis in the Metascape platform, 3 clusters (MCODE1, MCODE2, and MCODE3) were identified and extracted from the PPI network of the candidate targets. Enrichment analysis revealed that genes in the MCODE1 cluster were mainly enriched in the interleukin signal pathways, and the MCODE2 was associated with the leptin signaling pathway and PID telomerase pathway. Genes in cluster MCODE3 were primarily associated with cytokines and inflammatory response (Figure 3 B).

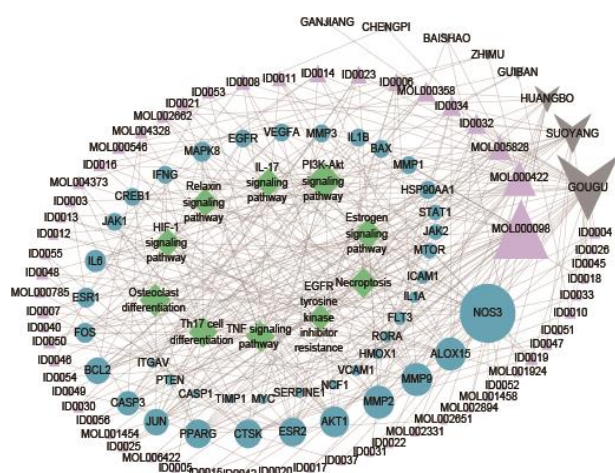


**Figure 3:** Enrichment analysis of the 85 candidate targets of HQW against OP. (A) The plot of the top 20 enrichment terms of these candidate targets. (B) MCODE analysis results in three main clusters from the PPI network of the candidate targets, namely MCODE1, MCODE2, and MCODE3

### Constructed herb-component-target-pathway network

To identify the hub targets and related components, the top 10 enriched non-disease-related pathways were selected to construct an H-C-T-P network, as shown in Figure 3. In addition, the PPI network was merged into the H-C-T-P network. Topological analysis was performed on the network, and the size and color

of target nodes were reset according to the degrees. It was revealed that NOS3, ALOX15, and MMP9 were the top 3 targets with the highest degree, indicating the important role of these genes in the effects of HQW on OP. Quercetin (MOL000098) exhibited the greatest degree of anti-OP activity, as confirmed by a comprehensive body of research. Signal transduction pathways, such as osteoclast differentiation, PI3K-Akt signaling pathway, and Th17 cell differentiation, were included in the network and were demonstrated to be associated with OP pathogenesis.



**Figure 4:** Herb-compound-target-pathway network of HQW against OP. The grey nodes, purple triangles, blue circles, and green rhombus represent herbs, active components, targets, and pathways, respectively

### Potential regulation of osteoclast differentiation by HQW

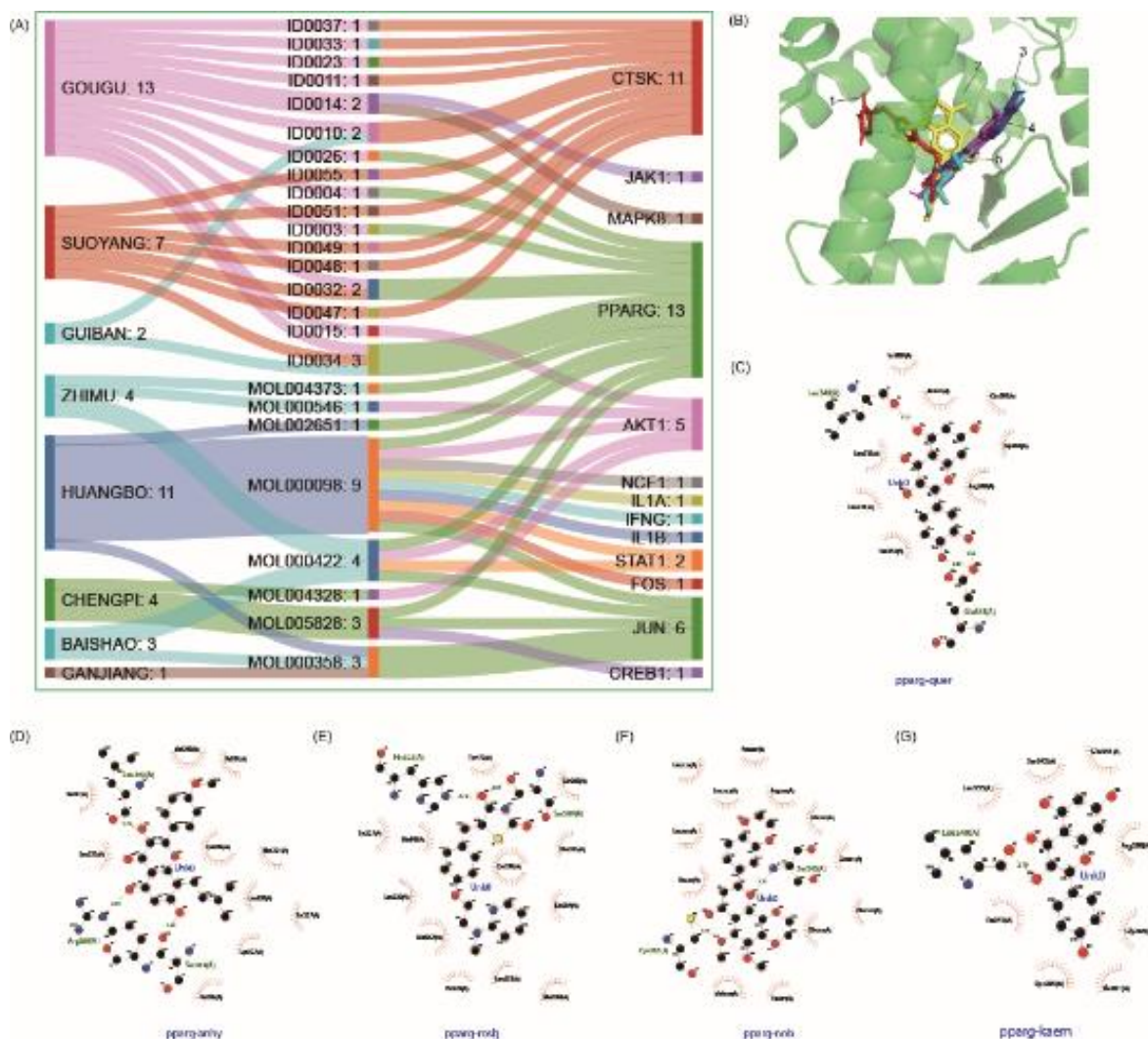
Given the close relationship between osteoclast and OP, the connections among the herbs, active components, and targets involved in the osteoclast differentiation were illustrated via a Sankey diagram (Figure 5 A). It was demonstrated that PPARG was the most connected target, followed by CTSK and JUN. MOL000098 was the most active component targeting the 9 genes related to osteoclast differentiation. The binding poses and binding affinities of PPARG with 4 active components (anhydroicaritin, quercetin, nobiletin, and kaempferol) were analyzed. Rosiglitazone, a PPPARG agonist, was used as a reference. As shown in Figure 5 B, the 3D complex showed binding poses of PPARG with five compounds in the ligand-binding pocket. Additionally, hydrogen bonding and van der Waals forces in the PPARG-ligand complexes were observed (Figures 5 C to G). Table 1 shows the binding energy and H-bonds in the PPARG-ligand complexes. Anhydroicaritin has the closest

affinity and hydrogen-bonding pattern to the reference agonist, suggesting that it might exert the same functions as rosiglitazone on PPARG. These data indicated that PPARG might interact with these active components to form compact complexes, thereby regulating osteoclast differentiation and preventing OP.

## DISCUSSION

Osteoporosis, a frequent form of secondary osteoporosis, can be linked with a heightened danger of fracture in many patients. *Huqian Wan* has been clinically demonstrated to be effective in the treatment of OP, as it is able to enhance murine trabecular bone structure to the normal level in an ovariectomized rat model of OP, as well as increase bone density, bone mass, and transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) levels in osteoblasts. Wang *et al.* recently reported that HQW reduced the level of serum interleukin-6 (IL-6) and alkaline phosphatase (ALP) in an ovariectomized rat model of OP while increasing the level of serum osteoprotegerin (OPG) [11]. However, the clinical application and formulation optimization of HQW are hindered by the insufficient understanding of its pharmacological mechanism against OP. Accordingly, this research utilized a network pharmacology-based approach to explore the potential molecular mechanism of the anti-OP effects of HQW in this study.

In this study, 128 active components in HQW were identified, and found 85 candidate targets of HQW with potential anti-OP effects. Interleukin-6 and AKT serine/threonine kinase 1 (AKT) were in the closest connection with other candidate targets in the PPI network, suggesting that these two targets of HQW might have strong anti-OP effects. Direct evidence for the regulation of IL-6 has been provided by Wang *et al* [11]. Additionally, single nucleotide polymorphism (SNP) rs1800796 in IL-6 was associated with an increased risk of OP. Further, accumulating evidence has reported the regulation of IL-6 cytokines in osteoblasts, osteoclasts, bone-resident osteocytes, and cartilage cells [12], IL-6 is thus a potential target for OP therapy. The AKT is an important serine-threonine protein kinase and participants in various biological processes. Multiple AKT-related signal pathways are extensively involved in the anti-OP effects of drugs, mediating osteoblast and osteoclast differentiation [13]. Nitric oxide synthase 3 (NOS3), an enzyme involved in the synthesis of nitric oxide, was found to be the most connected target in the H-C-T-P network, suggesting its crucial role in the anti-OP effects of HQW.



**Figure 5:** Regulation of HQW in osteoclast differentiation. (A) The Sankey diagram illustrated the herb-compound-target connections of HQW in osteoclast differentiation. (B) The 3D complex of PPARG with active compounds. Numbers 1 to 5 in the 3D diagram of the protein-ligand complex represent rosiglitazone, anhydroicaritin, quercetin, nobiletin, and kaempferol, respectively. (C-G) 2D diagrams of PPARG with active compounds which showed the hydrogen bonding and Van der Waals forces in the protein-ligands complex

**Table 1:** Binding affinity and H-bond details in PPARG-ligands complex

Compound	Binding energy	H-bond details	Distance
Anhydroicaritin	-7.77	Leu340	2.74
		Arg288	3.3
		Ser289	2.6
Kaempferol	-6.69	Leu340	2.79
		Nobiletin	-7.19
Quercetin	-6.36	Cys285	3.25
		Leu340	3
		Glu343	2.59
Rosiglitazone	-7.96	Glu343	2.87
		His323	3.12
		Ser289	2.89

Nitric oxide (NO), a signaling molecule synthesized by NOS3, has been shown to inhibit osteoclastogenesis and reduce bone resorption via down-regulating receptor activator of nuclear factor KappaB (RANKL)/OPG [14]. Several studies have been conducted to reveal the

contribution of NOS3 SNPs to the susceptibility of OP, yet the results have been inconsistent. Further multi-center investigations are thus needed.

As demonstrated in the H-C-T-P network, the top 3 hub active components were quercetin, kaempferol, and nobiletin. Quercetin possesses a wide spectrum of biological effects such as anti-cancer, anti-oxidative, and bone-sparing effects. In an OP mice model, oral intake of quercetin or its derivatives has been shown to inhibit bone loss [15]. Quercetin in combination with vitamin E, ameliorated ovariectomy-induced OP via modulating autophagy and apoptosis in osteoclasts. Additionally, quercetin attenuated OP induced by testosterone deficiency via regulation of glucose metabolism as well as inhibition of lipid metabolism. Kaempferol is a dietary bioflavonoid with osteoprotective effects. It has been reported to promote osteogenic differentiation of bone marrow mesenchymal stem cells (BMSC), and ameliorate OP by downregulating miR-10a-3p and upregulating C-X-C motif chemokine ligand 12 (CXCL12). Kaempferol could also induce osteoblast differentiation via the WNT/ $\beta$ -catenin signaling pathway and promotes bone formation in part via the mTOR signaling pathway [16].

Nobiletin, a polymethoxy flavonoid, has been found to exhibit dual-inhibitory effects on bone resorption and bone loss. Specifically, it was demonstrated to restrain bone resorption via inhibiting NF $\kappa$ B-dependent prostaglandin E synthesis in osteoblasts, and it was also shown to inhibit bone loss via regulating RANKL-induced regulation of osteoclastogenesis. In order to address the lack of hydrophilicity and cytotoxicity of nobiletin, a recent study developed a new delivery system (nobiletin-loaded micelles), which was demonstrated to attenuate bone loss and improved bone density in ovariectomized mice [17]. However, more research need to be conducted to identify the osteoprotective effects of the active components in HQW and elucidate the synergistic mechanism of these active components in treating OP.

The results highlighted the involvement of interleukin signals and inflammatory response in the anti-OP effects of HQW. Recent evidence has established a connection between OP and inflammation. A range of pro-inflammatory cytokines, including IL-6, tumor necrosis factor (TNF- $\alpha$ ), IL-8, interleukin-1 $\beta$  and interleukin-17, have been found to be involved in both physiological and pathological osteoporosis, acting as both bone resorption stimulators and bone formation inhibitors. A variety of drugs attenuate OP via inhibiting inflammation response, suggesting the crucial role of inflammation response inhibition in the treatment of OP. Pathway enrichment analyses suggested that the estrogen signaling pathway participated

in the anti-OP effects of HQW. The estrogen receptor 1 (ESR1) plays an integral role in controlling bone metabolism. It has been observed that a surplus of estrogen can lead to osteoporosis. It was proposed that the increased osteoclastogenesis or decreased osteoblastogenesis induced by ESR1 downregulation in B cells are associated with postmenopausal OP [18].

Osteoporosis is usually considered as a manifestation of imbalance between osteoblasts bone formation and osteoclasts resorption, in which the regulation of osteoclast differentiation plays a crucial role in maintaining the balance. It has been confirmed that the excessive promotion of osteoclast differentiation is the primary cause of cadmium-induced osteoporosis [13]. In the current study, the candidate anti-OP targets of HQW were also enriched in osteoclast differentiation, and Sankey diagram highlighted the important role of peroxisome proliferator-activated receptor-gamma (PPARG) in regulating osteoclast differentiation by HQW. Peroxisome proliferator activated receptor gamma (PPARG) is an essential transcription factor for adipogenesis, and has been implicated in the pathogenesis of OP. Notably, PPARG is also critical for sclerostin expression in osteocytes, and its deletion leads to decreased bone mass and marrow adiposity, as well as TZD-induced bone loss. Molecular docking analysis showed that the binding affinities and binding positions of anhydroicaritin, quercetin, nobiletin, and kaempferol with PPARG were close to those of rosiglitazone, suggesting that these components may act as PPARG agonist. However, direct evidence of the influence of these active components on PPARG is lacking.

### Limitations of this study

There are several limitations in this study. First, the mechanism of HQW against OP was only investigated using a systematic informatics approach, without any experimental validation *in vitro* and *in vivo*. Secondly, due to the confounding factors such as databases and prediction software, the identification of the active components and targets of HQW was not comprehensive enough.

### CONCLUSION

This study identifies the active components and the candidate targets associated with anti-OP effects in HQW, and proposes that the anti-OP activity of HQW is linked to the modulation of multiple signal transduction pathways and

osteoclast differentiation, with PPARG being identified as a potential mediator of the anti-OP effects of HQW through regulating osteoclast differentiation. It provides preliminarily evidence of the underlying molecular mechanism of HQW in treating OP. Further validation of these effects both *in vitro* and *in vivo* will be necessary.

## DECLARATIONS

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None provided.

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### Ethical approval

None provided.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Mengchuan Zhuo originated the research concept, designed the study, collected and analyzed the data, and wrote the draft of the manuscript; Zhoufeng Song validated the research results, and read and revised the manuscript. All authors read and approved the manuscript for publication.

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