Research of the molecular mechanism of Hai Honghua medicinal liquor in the treatment of fracture based on network pharmacology combined with molecular docking

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Abstract

Background: Hai Honghua medicinal liquor (HHML), a famous hospital formula composed of 19 traditional Chinese medicines, has been successfully applied in treating soft tissue injury, fresh closed fracture, limb dysfunction after fracture healing, shoulder, neck and leg pain, knee joint pain and other clinical multiple diseases for 30 years in clinical. However, research on the material basis of HHML for the treatment of fracture healing-related disorders is still in a gap. Therefore, it is particularly important to explore the active ingredients, core targets and potential pharmacological mechanisms of HHML to promote fracture healing. Methods: We screened the core active components of each traditional Chinese medicine in formula and its action targets through the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform and Encyclopedia of Traditional Chinese Medicine database. The fracture related targets were retrieved from several different public databases, including GeneCards, Online Mendelian Inheritance in Man, DisGeNET and Therapeutic Target Database. Bioinformatics analysis to obtain key bioactive components, underlying targets and signaling pathways, containing the Venn diagram of the intersection with components and diseases gene targets, protein–protein interaction, as well as the Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes analysis, and finally molecular docking. Results: A total of 249 bioactive ingredients of HHML and 325 HHML-fracture-related targets were screened. The network analysis revealed that quercetin, luteolin, kaempferol, Licochalcone A, naringenin and 8-isopentenyl-kaempferol may be potential candidate agents. Multiple targets are involved including TP53, MAPK3, STAT3, AKT1, MAPK1, HSP90AA1, ER81 and PIK3CA may be closely linked targets. PI3K-AKT signaling pathway may play a significant role of HHML in treatment of fracture. What's more, molecular docking suggested that 8-isopentenyl kaempferol, glycyrrhiza chalcone A, and naringenin bound to AKT1, PIK3CA, and ER81, respectively, exhibiting lower energy and more stable characteristics. Conclusions: The findings indicate the potential active ingredients, target proteins and molecular mechanisms of HHML for the treatment of fractures to provide the exact idea for the next research on the mechanism of action of HHML formula for fracture treatment.

Keywords: Hai Honghua medicinal liquor; fracture healing; network pharmacology; molecular docking; PI3K/AKT signal pathway
Introduction

Fracture, a damaged disease characterized by the integrity and continuity of the bone, is increasingly common with advancing society. This increase in bone fracture, results in violent injuries such as traffic accidents. In the elderly, low bone mass and a history of fractures are strong risk factors for skeletal fractures [1]. In the treatment of fractures, there are often complications such as infection, pain, mal-union, nonunion or delayed union, which seriously affect the quality of life of patients [2–4]. Therefore, the treatment of fractures is also an urgent clinical problem that needs to be paid attention to. Fracture healing, bone regeneration, and bone growth integrity, that is, to restore the composition, physiological structure and biological function of the body’s cells to a normal state [5]. There are four structures at the fracture site, namely cortical bone, periost, bone marrow, and external tissue, which are all involved in the fracture healing process. In the process of fracture healing, the role of each physiological structure varies according to the biological level in the tissue, such as growth factor level, thyroid hormone level, blood supply level, pH value, oxygen tension, electrolyte environment and fracture site stability [6]. Fracture healing is a complex physiological process that mainly involves four biological processes, including inflammatory response, soft callus formation, endochondral ossification and bone remodeling [7, 8]. Fully understanding the physiological basis of fracture healing, which will be reflected in the process of tissue healing, lays a theoretical foundation for us to choose the appropriate treatment strategy.

Hai Honghua medicinal liquor (HHML) is an in-hospital preparation, which has been successfully used in clinical practice for more than 30 years and has good effect on fracture healing. The formula contains 19 herbs, of which Aralia chinensis L is also called as orthopaedic medicine, and it can dispel wind and remove dampness, and relieve pain, and is widely used in the field of orthopaedics and traumatology. Studies have reported that the active ingredients from Aralia chinensis L modulate the levels of inflammatory factors, inhibit apoptosis, and attenuate endothelial dysfunction by activating PI3K/Akt signaling [9]. Carthamus tinctorius L is especially good at invigorating the blood, dispelling blood stasis and has a variety of pharmacological activities. Safflower active ingredients have been studied in the treatment of cancer [10], diabetes [11] and other diseases, with anti-inflammatory, antioxidant, anti-apoptotic, protection of cardiovascular function and other rich pharmacological activities [12, 13]. In particular, for the mouse ischemic necrosis model, safflower was able to enhance the bone density of the femoral head, effectively promote bone formation and prevent osteonecrosis in mice [14]. A large body of accumulating evidences suggested that traditional Chinese medicine (TCM) has already been utilized to cure various types of orthopaedic illnesses such as inflammation, fracture, osteopenia, and osteoporosis [15]. According to the basic theory of TCM, post-operative swelling after fracture belongs to the category of “blood stasis”, which is caused by blockage of tendons and veins, stasis of blood outside the veins and suspension of fluid. In addition, the formula contains several Chinese herbs with the efficacy of activating blood circulation and removing blood stasis, reducing swelling and pain, and relaxing tendons and activating joints, which can enhance its efficacy on soft tissue injury, fresh closed fracture, limb dysfunction after fracture healing, shoulder, neck, lumbar and leg pain, knee pain and other clinical multifarious diseases when used together, and is of profound significance for clinical promotion and application research.

In recent years, network pharmacology methods are widely used to study TCM formula, to screen multiple components corresponding to targets in a high-throughput manner, sequence disease-related genes, establish drug-gene-target-disease networks, and provide research ideas for the development of new drugs [16]. Specifically, the usual steps of a network pharmacology study include searching for key components and targets with the help of existing databases, software analysis to construct the network, and experimental studies were conducted to make the prediction results more reliable [17, 18]. The combination of network pharmacology and molecular docking technology [19] predicts the potential mechanism of Chinese medicine for treating diseases, provides a reference for the screening of active ingredients in Chinese medicine, and can even be said to lay the foundation for the development of modernization of TCM.

In summary, network-based pharmacology and combined molecular docking techniques were used to explore the potential targets and molecular mechanisms of HHML for the treatment of fractures and to lay the foundation for further research as well as to promote the clinical use of the medicinal liquor. The workflow is shown in Figure 1.

Materials and methods

Materials


Network pharmacology analysis


Figure 1 The workflow of this study. HHML, Hai Honghua medicinal liquor; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; ETCM, Encyclopedia of Traditional Chinese Medicine; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; PPI, protein protein interaction.
Chinese Medicine (ETCM) (http://www.tcmip.cn/ETCM/index.php/Home/index/), were used for searching herb compounds; the disease targets were obtained from GeneCards (https://www.genecards.org/), Online Mendelian Inheritance in Man (https://omim.org/), DisGeNET (https://www.disgenet.org/) and Therapeutic Target Database (http://db.idrblab.net/ttd/) databases; the overlapping gene targets between TCM compounds and diseases were researched in VENN2.1 (https://bioinfose.cn.csic.es/tools/venny/index.html) online database; STRING (https://cn.string-db.org/) database was used to form protein-protein interaction (PPI) network program; DAVID bioinformatics resources (https://david.ncifcrf.gov/) integrates the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Gene Ontology (GO) biological process; visualization of target data by Cytoscape 3.9.1 software.

Target prediction. The main components of 19 herbal medicines contained in HHML were retrieved from the TCMSP and ETCM public databases. The metabolic process of drugs in the body includes absorption, distribution, metabolism and excretion, which is the key to evaluate the pharmacokinetics. According to the reported literature to screening bioactive components, oral bioavailability (OB) value is usually selected OB ≥ 30%. A drug-likeness (DL) index ≥ 0.18 is used as a criterion for screening drug activity. The active components of HHH resulted from this.

Fracture target genes were obtained from four databases, namely, GeneCards, Online Mendelian Inheritance in Man, DisGeNET and Therapeutic Target Database. These databases are the discovery platform containing many publicly available collections of genes and variants associated with human diseases. The search term comprised “Fracture”, “bone fracture”, “catagma”, “bone defect”, “tibia and fibular fractures” and “bone destruction”. Disease targets retrieved from different databases are merged into one file.

Intersection of active ingredient targets and disease targets were collected by using VENN2.1 online database. The overlapping genes are presented intuitively in the form of Venn diagram.

PPI network construction. The interrelated proteins in the PPI network may have the same or similar functions, which helps to understand the targets and molecular mechanisms of HHML for fracture healing from a systems perspective. The STRING database allows us to obtain association maps and data files between the proteins in preparation for the next visualization step. Moreover, it is used for searching protein-protein interaction network, digging the core regulatory genes and analyzing the differential genes. The parameters in STRING system were set as follows: upload 325 targets of HHML related to fracture, select “Homo sapiens” as the species, set the minimum required interaction score to 0.9, remove the free protein targets, export the data in tab-separated values format, and then import the obtained data files into Cytoscape 3.9.1 for PPI network analysis.

GO and KEGG enrichment analysis for HHML-fracture-related targets. The DAVID analysis tool can provide systematic biofunctional annotation information for large-scale gene or protein ID. Uploading the overlapping gene list into the database, choosing official gene symbol and Homo sapiens conducted analysis. The data of biological process (BP), cellular component (CC) and molecular function (MF) were downloaded respectively. At the same time, KEGG pathways also were gained. The top 10 enriched genes in terms of count and P value were selected for GO analysis and the top 30 for KEGG analysis.

Construction of network and analysis. In order to investigate the action mechanism of active ingredients of HHML used promote bone fracture healing. We constructed a “components-disease-target-pathway” network by using the Cytoscape 3.9.1 software package. The network shows systematic interaction of the complicated relationships among ingredients, genes, pathways and diseases. The complex relationships are a key point in the subsequent studies.

Molecular docking

In order to explore the potential target and drug activity of small drug molecules, molecular docking technology analysis was carried out. Molecular docking is an effective means of accurately predicting the appropriate target of an active ingredient based on its binding capacity and binding site to large molecule protein receptors and small molecule ligands. Based on the results in the PPI network, suitable large molecule protein receptors were screened in the PDB online database and 3D structures of small molecule compounds were mapped. Docking was performed using AutoDock tools software to analyze the binding ability of the docked protein receptors to the small molecule ligands.

Results

Network pharmacology analysis

Active ingredients. The chemical composition of each herbal medicine contained in the prescription of HHML was searched according to the TCMSP, ETCM online database. According to the screening conditions reported in the literature, OB ≥ 30% and DL ≥ 0.18, a total of 151 chemical components of HHML were obtained. Then, the common components of herbal medicines were removed and 80 chemical components were screened out.

Components and disease target screening. A total of 1532 initial targets of 80 chemical components of HHML were obtained through the UniProt online gene database. In addition, 9318 fracture-related targets were obtained from the 5 databases. As shown in the Figure, the components were intersected with the disease targets to obtain 325 relevant common targets.

Figure 2 Venn diagram. Intersection of HHML and fracture-related targets. HHML, Hai Honghua medicinal liquor; OMIM, Online Mendelian Inheritance in Man; TTD, Therapeutic Target Database; CTD, Comparative Toxicogenomics Database.
PPI network. A total of 325 intersection targets for HHML treatment of fractures were uploaded to the STRING database with 325 nodes and 1321 edges to obtain the PPI network. The node selection condition is set to highest confidence 0.9. To analyze the protein-protein interactions, PPI data were imported into Cytoscape 3.9.1 software to construct visual network diagrams (Figure 3). The selection was made according to the magnitude of the degree value, and also combined with the magnitude of the betweenness and closeness value to make the graph, and the top 10 of degree value were selected and summarized in Table 1. In the PPI network, large nodes and red color represent higher degree value; conversely, small nodes and light color represent lower degree value. The central targets are AKT1, TP53, MAPK3, and STAT3, which may deserve our attention in the role of HHML in treating fracture healing.

Enrichment analysis. To elucidate the potential gene functions and enrichment pathways of HHML for fracture treatment by GO enrichment analysis and KEGG pathway enrichment analysis. The GO analysis includes BP, CC, and MF. Specifically, 1506 GO terms were obtained, including 1111 BP terms, 148 CC terms and 247 MF terms. The top 10 significantly enriched terms according to the highest enriched gene counts of BP, CC and MF were visualized in a bar plot chart in Figure 4A. The results suggest that the relevant targets of HHML for fracture healing may be primarily enriched in terms of drug response to (GO: 0042493), response to xerogenic stimulus (GO: 0009410), aging (GO: 0007568), and other biological processes; cytosol (GO: 0005829), macromolecular complex (GO: 0032991), caveola (GO: 0005901), and other cellular components; enzyme binding (GO: 0019899), identical protein binding (GO: 00428001), protein binding (GO: 0005515), and other molecular function.

The KEGG analysis was explored relevant signaling pathways and laid the foundation for research into the mechanisms of HHML therapy for fracture healing. A total of 201 signaling pathways, in which the top 30 enriched pathways in terms of gene number as well as P value are presented as bubble charts (Figure 4B), suggesting that HHML plays a crucial role in the treatment of fracture healing through multiple targets and pathways. As seen by the KEGG results, the

![Figure 3 The PPI network of HHML and fracture targets. Nodes represent proteins and the colors from green to blue to red represent the degree of binding between proteins. The linkage indicates that there is an association between the two proteins. HHML, Hai Honghua medicinal liquor; PPI, protein-protein interaction.](https://www.tmrjournals.com/im)

<table>
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<tr>
<th>Gene symbol</th>
<th>Target name</th>
<th>Degree</th>
<th>Betweenness</th>
<th>Closeness</th>
</tr>
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<td>TP53</td>
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<td>9972.382983</td>
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<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
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<td>3977.551297</td>
<td>0.061107434</td>
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<tr>
<td>AKT1</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
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<td>5996.353375</td>
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<tr>
<td>MAPK1</td>
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<td>HSP90AA1</td>
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<tr>
<td>ESR1</td>
<td>Estrogen receptor</td>
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<td>4243.268507</td>
<td>0.061419069</td>
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<tr>
<td>PIK3CA</td>
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<td>MAPK14</td>
<td>Mitogen-activated protein kinase 14</td>
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HHML, Hai Honghua medicinal liquor.
first ranked term is PI3K-Akt signaling pathway. Interestingly, we found that the hub genes of AKT1 and PIK3CA, mentioned above, coincide with the pathway targets.

**Components-target-pathway-drug-disease network analysis.** To further discover the more details related to the mechanism, the 80 components and top 10 pathways related to 1855 targets were selected to construct a "components-target-pathway-drug-disease" network. As shown in Figure 5, a multi-component, multi-target complex network was obtained, with different nodes distinguished by different colors. In this network, the main active ingredients were obtained through its degree value. The 6 chemical components with strong correlation were selected as shown in Table 2.

**Molecular docking analysis.** We selected 3 candidate protein receptors that are closely related to the KEGG pathway including AKT1, PIK3CA, ESR1, were analyzed molecular docking with 3 candidate chemical components (8-Isopentenyl-kaempferol, Licochalcone A, naringenin). The processed proteins and small molecule ligands were imported into AutoDock software, docked separately, and 10 docking images were obtained. The best image (lowest binding energy) was saved as "pdbqt" format, and then imported into pymol for graphing (Figure 6). As can be seen from the data in the Table 3, the main components of HHML have low binding energy and good stability with important targets, respectively. Obviously, naringenin has a good binding ability to these targets.

![Figure 4](image)

**Figure 4** The results of GO enrichment analysis and KEGG pathway enrichment analysis. (A) Top 10 GO enrichment analysis of hub genes. (B) Top 20 KEGG pathway of hub genes. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function.

![Figure 5](image)

**Figure 5** Components-target-pathway-drug-disease network of HHML. The outermost circle in yellow represents 80 components of HHML, the inner circle represents the gene target, the middle square indicates that there are 10 pathways, the disease is indicated in the middle, and the name of the medicine wine is indicated by a red polygon. HHML, Hai Honghua medicinal liquor.
Table 2 The active ingredients and their properties and structures

<table>
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<tr>
<th>Mol ID</th>
<th>Molecule name</th>
<th>OB</th>
<th>DL</th>
<th>Structure</th>
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</thead>
<tbody>
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<td>MOL000098</td>
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<td>MOL000006</td>
<td>luteolin</td>
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<td>0.25</td>
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<tr>
<td>MOL000422</td>
<td>kaempferol</td>
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<td>0.24</td>
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<td>MOL000497</td>
<td>Licochalcone A</td>
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<td>0.29</td>
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<tr>
<td>MOL004328</td>
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<td>38.04</td>
<td>0.39</td>
<td><img src="8-isopentenyl-kaempferol.png" alt="Structures" /></td>
</tr>
</tbody>
</table>

OB, oral bioavailability; DL, drug-likeness.

Table 3 The binding energy of 3 core targets and their interacting components.

<table>
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<tr>
<th>Target</th>
<th>PDB ID</th>
<th>Components</th>
<th>Auto-dock energy</th>
</tr>
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<tr>
<td>AKT1</td>
<td>4gv1</td>
<td>MOL000497</td>
<td>−3.82</td>
</tr>
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<td></td>
<td></td>
<td>MOL004328</td>
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<td>−6.47</td>
</tr>
</tbody>
</table>

PDB, Protein Data Bank.
In clinical practice, HHML is used to treat the more common fresh closed tibia fractures and the improvement of limb dysfunction after fracture healing, and the molecular mechanisms by which it exerts its efficacy deserve more in-depth study. This traditional Chinese medicine formula contains 19 herbs with complex and diverse chemical compositions corresponding to a large number of action targets. We mined the ingredients and related disease targets through appropriate online databases, filtered out duplicate values, and finally obtained 80 related ingredients. Next, the protein interaction network, target pathway analysis and molecular docking were used to obtain the main 3 chemical components, including 8-isopentenyl-kaempferol, Licochalcone A and naringenin. 8-Isopentenyl-kaempferol, as an important flavonoid, is present in many traditional Chinese medicines and has various biological activities, also known as desmethylcaritin. It has been reported that 8-isopentenyl-kaempferol inhibits inflammatory gene expression and suppresses redox-sensitive activation of the PI3K/PTEN/Akt pathway in macrophages treated by H$_2$O$_2$ [20]. In addition, Chio et al. showed that 8-isopentenyl-kaempferol plays an important role in bone formation using MC3T3-E1 cell model, promoting differentiation and proliferation of osteoblasts as well as inducing bone nodule formation with anti-osteoporotic potential [21]. Kim et al. reported that Licochalcone A induced osteogenic differentiation in in vitro studies, and in vivo studies demonstrated its ability to promote osteogenesis and exhibit potential therapeutic abilities for bone-related diseases [22]. Naringenin is a potential anabolic agent for the treatment of bone loss through the regulation of osteogenesis, osteoclastogenesis and macrophage polarization, linked to multiple molecular mechanisms such as PI3K/Akt and it promotes bone formation while inhibiting bone resorption, thereby achieving skeletal protection [23, 24]. The potential active ingredients obtained from the results of this study are consistent with existing studies reporting an important role in the intervention of fracture disorders.

The fracture injury targets were retrieved in different databases as shown in Figure 2. We found that fracture and HHML have 325 common targets and these targets may be potential targets for the treatment of fracture healing. To explore disease-drug key targets, protein-protein interactions were resolved by constructing PPI networks. The outcomes revealed that TP53, MAPK3, STAT3, AKT1, MAPK1, HSP90AA1, RELA, ESR1, PIK3CA, MAPK14, especially AKT1, TP53, MAPK3 and STAT3, may be the core targets. Akt1 has been identified as a key regulator of osteoblasts and osteoclasts, maintaining bone mass by promoting their differentiation and survival [25]. These studies reported provide us with a theoretical basis.

To understand to which cell functions the genes we studied were enriched and to which signaling pathways the differential genes were enriched, GO analysis and KEGG enrichment analysis were used to obtain relevant information. KEGG results yielded 201 pathways, excluding apparently unrelated pathways such as pathways in cancer, lipid and atherosclerosis, and then obtained the highest ranked PI3K-Akt signaling pathway based on the count of genes and P-value. The results implied that PI3K-Akt signaling pathway may be a noteworthy molecular mechanism in the treatment of fracture disease by HHML. There are relevant literature reports connected with the regulation of PI3K/Akt signaling pathway in promoting osteoblast proliferation and differentiation as well as in promoting the osteogenic process [26]. This pathway was enriched by network pharmacological analysis to provide ideas for our subsequent experimental studies. Exploring the molecular mechanism of HHML for fracture healing became our primary task to address and lay the material foundation for clinical studies.

To search the mechanism of action of HHML in the treatment of fracture, we molecularly docked the key protein targets to the possible active ingredients separately and found that not all the compounds in Table 2 had good binding ability. Finally, three potential active
ingredients (8-Isopentenyl-kaempferol, Licochalcone A and naringenin) were identified based on the good binding ability of the protein to the compound molecules. The docking results show that AKT1, PIK3CA and naringenin have the lowest binding energy. These results suggest a possible molecular mechanism and the AKT1 and PIK3CA targets echoed the REGG enrichment results.

The study on the mechanism of action of HHML for fracture disorders is still inadequate. Our study only explores the role of HHML in fracture treatment using network pharmacology and molecular docking. However, the existing public databases contain relevant information that lacks timeliness, does not update the latest data in a timely manner, and lacks reliability for the obtained data. Although 8-Isopentenyl-kaempferol, Licochalcone A, and naringenin have been considered as 3 vital active constituents in the treatment of fracture healing by HHML, in this paper, they do not fully represent HHML formulas. Therefore, it is necessary to consider in vivo animal studies on the effects of fracture healing and in combination with in vitro studies on the mechanisms of osteogenesis to further substantiate the study in several aspects. Due to the lack of reports on the mechanism of HHML formula in treating fracture healing, we have conducted relevant research for the first time, and continue to further verify that the research is considered significant, laying a theoretical foundation for clinical use and promotion.

Conclusion

This study was based on bioinformatics-related techniques to investigate the chemical composition targets of 19 herbs in HHML formulas and the potential mechanisms for treating fractures. 8-Isopentenyl-kaempferol, Licochalcone A, and naringenin may be the main active components of HHML, and the protein targets PIK3CA, AKT1, and ESR1 may be potential therapeutic targets of HHML for fracture healing. The molecular mechanism may be closely related to the PI3K-AKT signaling pathway, with a focus on this pathway. In addition, this study lays some foundation for the study of the mechanism of action of HHML to promote fracture healing.

References


