Traditional Medicine Research
doi: 10.12032/TMR20210603232

Traditional Chinese Medicine

Systematically characterized mechanism of Yanhusuo powder ingredient absorbed in rat plasma for treatment osteoarthritis via UPLC-Q-TOF/MS with UPLC-MS/MS and network pharmacology

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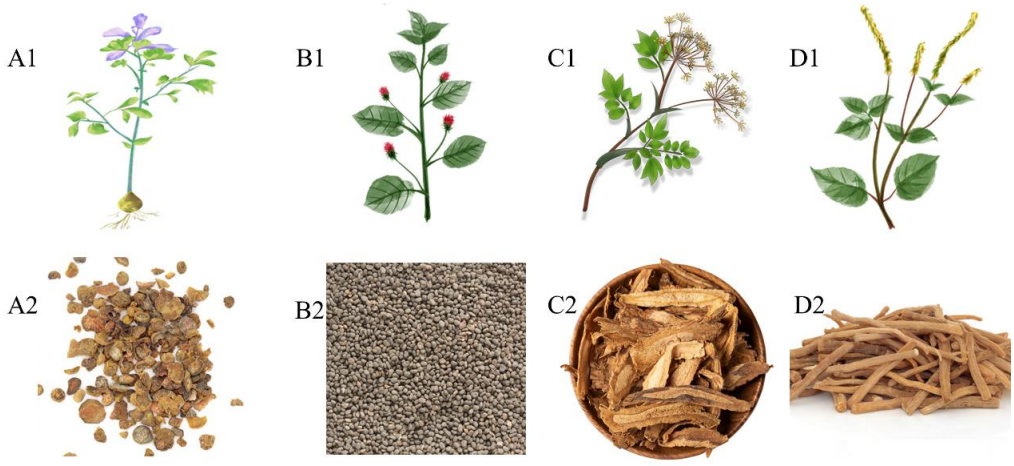
Highlights

Thirty-four types of in vitro components and 20 types of blood prototype components were identified in Yanhusuo powder. Components such as protopine and dehydrocorybulbine may regulate the PI3K-AKT and mitogen-activated protein kinases signalling pathways by acting on AKT1 and TNF targets, thus potentially having a role in the treatment of osteoarthritis.

Tradition

Yanhusuo powder (also known as Xuanhusuo powder) was first recorded during the Ming Dynasty in the *Prescriptions for Universal Relief*, which was written by Zhu Su and published in 1390 C.E. Yanhusuo powder is composed of *Corydalis yanhusuo W. T. Wang*, *Psoralea corylifolia L*, *Achyranthes bidentata Bl*. and *Angelica sinensis* (*Oliv*.) *Diels* in a ratio of 1:1:1:1. Yanhusuo powder has the comprehensive effects of activating blood circulation and relieving pain. Nowadays, Yanhusuo powder is widely used as a basic formula for the clinical treatment of osteoarthritis. The main components of Yanhusuo powder include flavonoids, coumarins, alkaloids and saponins, as determined in our previous study; however, serum pharmacochemistry studies, as well as the relevant pharmacological mechanisms, have yet to be completely clarified.



Abstract

Background: Yanhusuo powder, also known as Xuanhusuo powder, is a long-standing Chinese herbal formula mainly used in the treatment of osteoarthritis. Although the clinical effectiveness of Yanhusuo powder has long been acknowledged, its mechanism of action and bioactive components remain unknown. **Methods:** A novel analytical method combining the use of ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry and ultra-performance liquid chromatography-triple quadrupole mass spectrometry was applied to profile the formula and absorbed prototype components in plasma after oral administration of Yanhusuo powder. Then, the absorbed constituents were subjected to network pharmacology to predict targets and pathways. AutoDock software was then used for molecular docking studies to screen for potential pharmacodynamic substances. **Results:** A total of 34 in vitro formula components and 20 in vivo prototype compounds from the various relevant species were successfully separated and identified for the first time. Compound-target-pathway analysis revealed that 20 absorbed constituents, 42 target genes and 42 pathways are probably related to the efficacy of Yanhusuo powder against osteoarthritis. The efficacy of Yanhusuo powder mainly involves AKT1, fibronectin 1 and matrix metalloproteinase 9 targets and apoptosis, as well as PI3K-AKT and mitogen-activated protein kinases signaling pathways. According to the results of the molecular docking studies, it can be preliminarily judged that protopine, dehydrocorybulbine and angelicin may be the pharmacologically active substances of Yanhusuo powder. **Conclusion:** The results provide a scientific basis for understanding the bioactive compounds and the pharmacological mechanism of Yanhusuo powder.
Keywords: Yanhusuo powder, Prototype components, Mass spectrometry, Network biology strategy

***Author contributions*:**

Yu-Xia Qu and Na Zhang helped with study design, data interpretation and writing the manuscript; Chen-Ning Zhang helped with data statistical analysis and writing the manuscript; Yu Sun helped data statistical analysis; Run-Hua Liu, Shi-Ting Ni, Yu-Ting Ding and Di Geng helped to animal experiment; Jie Luo and Yi-Kun Sun funded all experiments, analyzed and interpreted data.

***Competing interests*:**

The authors declare no conflicts of interest.

***Acknowledgments*:**

The work was supported by the National Natural Science Foundation of China (grant number: 81373942) and the Key Science and Technology Research Projects of Tibet Autonomous Region of China (XZ201801-GA-16).

***Abbreviations*:**

OA, osteoarthritis; TCM, traditional Chinese medicine; YHSP, Yanhusuo powder; UPLC-Q-TOF/MS, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography-triple quadrupole mass spectrometry; ESI, electrospray ionization; MRM, multiple-reaction monitoring; PPI, protein-protein interaction; KEGG, Kyoto Gene and Genome Encyclopedia; MAPK8, mitogen-activated protein kinase 8; mTOR, mammalian target of rapamycin.

***Citation*:**

Qu YX, Zhang N, Luo J, et al. Systematically characterized mechanism of Yanhusuo powder ingredient absorbed in rat plasma for treatment osteoarthritis via UPLC-Q-TOF/MS with UPLC-MS/MS and network pharmacology. *Tradit Med Res*. 2021;6(6):51. doi: 10.12032/TMR20210603232.

***Executive editor*:** Rui-Wang Zhao.

***Submitted*:** 12 April 2021, ***Accepted*:** 26 May 2021, ***Online*:** 17 June 2021.

doi: 10.12032/TMR20210603232

Background

Osteoarthritis (OA) is a degenerative joint disease that can lead to chronic structural damage of articular cartilage, which is caused by aging, obesity, strain and trauma [1]. The onset of OA is accompanied by long-term pain, stiffness, inflammation and other symptoms, which not only affect a patient's quality of life and bring huge economic losses, but also increase the mortality of the elderly and cause a great burden on society [2]. At present, there are three main ways to treat OA: joint replacement and non-drug and drug treatments, however, all of these methods have some limitations and the effects of long-term are not beneficial [3]. Therefore, searching for ideal drugs for treating OA is a hot spot in domestic and foreign research.

It has been acknowledged that traditional Chinese medicine (TCM) has a complex chemical system and ingredients absorbed into the blood are a critical component in its various therapeutic effects, which is known as serum or plasma pharmacochemistry theory [4, 5]. Based on the above theory, TCM treatments can be evaluated by analyzing drug-derived substance groups absorbed in the blood following oral administration of related treatments in order to identify potential pharmacologically active substances, a step which is essential in revealing the mechanisms of their clinical effects [6].

Yanhusuo powder (YHSP, also known as Xuanhusuo powder) [7, 8] was first recorded in the *Prescriptions for Universal Relief* during the Ming Dynasty; this bible was written by Zhu Su and published in 1390 C.E. YHSP is composed of *Corydalis yanhusuo W. T. Wang*, *Psoralea corylifolia L*, *Achyranthes bidentata Bl*. and *Angelica sinensis* (*Oliv.*) *Diels* in a ratio of 1:1:1:1. YHSP has the comprehensive effect of activating blood circulation and relieving pain. Nowadays, YHSP is widely used as a basic formula for the clinical treatment of OA. The main components of YHSP include flavonoids, coumarins, alkaloids and saponins, as determined in our previous study [9]. However, serum pharmacochemistry studies involving YHSP are yet to be conducted, and its pharmacological mechanisms have yet to be completely clarified. Therefore, an accurate, sensitive and reliable method for identifying the pharmacochemical compounds of YHSP in vivo are urgently needed in order to explore its mechanism(s) of action.

Modern mass spectrometry technology has been an effective and popular approach for exploring absorbed blood components and systematically revealing the "multi-targets, multi-components" mechanisms of complex herbal formulas [10]. Meanwhile, the network biology strategy integrates network theory and system biology theory to show the network of TCM components-targets-diseases in a visual way [11]. Molecular docking technology is a computer-aided drug design method that is popular for discovering lead compounds, screening effective components of TCM and discovering the basis of action of TCMs [12]. In our previous study, online websites were used to collect the ingredients of each flavor of YHSP and to predict their mechanisms [7]. At present, based on the theory of serum pharmacochemistry, mechanism predictions according to blood ingredients will make the predicted results more reasonable and credible.

This study aimed to characterize the absorption of YHSP in rat plasma and elucidate its mechanism of action in the treatment of OA. A strategy involving ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS) and combined with ultra-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-MS/MS) analysis was established to identify the compounds in YHSP. Subsequently, following the oral administration of YHSP, the more sensitive UPLC-MS/MS method was used to analyze the prototype components absorbed in rat plasma. Finally, network pharmacological analysis and molecular docking verification were performed on the accurately identified prototype components, in order to further understand the mechanism of action of YHSP in the treatment of OA. The credible results presented in our study will provide a scientific basis for YHSP in the supportive treatment of OA.

Materials and Methods

Ethics statement

All animal experiments were approved by the Animal Ethics Committee (BUCM-4-2021041301-2007) of the Beijing University of Chinese Medicine.

Chemicals and reagents

Standards of ferulic acid, ecdysterone, columbamine, tetrahydropalmatine, psoralen, angelicin, neobavaisoflavone, psoralidin and bakuchiol were purchased from Shanghai Yuanye Pharmaceutical Technology Co., Ltd. (Shanghai, China); the purity of all of these compounds was greater than 98.0%. *Corydalis yanhusuo W. T. Wang*, *Psoralea corylifolia L*, *Achyranthes bidentata Bl*. and *Angelica sinensis* (*Oliv.*) *Diels* were provided by the Beijing Tong Ren Tang Pharmaceutical Co., Ltd. (Beijing, China). The ingredients were identified by professor Sun Yikun (Beijing University of Chinese Medicine, Beijing, China). The entire plants and medicinal sites of four herbs of YHSP are shown in Figure 1. LC-MS grade acetonitrile was purchased from Fisher Scientific Co., Ltd. (Santa Clara, CA, USA) and used for analysis. Leucine enkephalin was purchased from Waters Technologies Co., Ltd. (Shanghai, China). Water was purified using a Milli-Q Plus water purification system

(Millipore Corporation, Billerica, MA, USA). Formic acid (98% purity), which was used as an ionization reagent in the mobile phase, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of YHSP decoction

YHSP decoction was prepared from the four herbs detailed in the original formula in a ratio of 1:1:1:1. The raw medicinal materials were pre-immersed in eight times their volume of distilled water for a half hour and then boiled for 30 minutes. Thereafter, six-time volumes of distilled water was added into the residue, which was decocted twice for 30 minutes. All collected supernatants were filleted through four layers of gauze and concentrated by a rotary evaporator to create an extract equivalent to 0.15 g/mL of raw medicine.

Animals

Six male Sprague-Dawley rats (8 weeks, 200 ± 20 g) were purchased from SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China). Animals were kept in an environmentally-friendly breeding room under controlled environmental temperature (24 ± 2 °C) and humidity (67 ± 1.5%) with a 12-hour dark-light cycle; food and drink were supplied ad libitum for 10 days until complete adaption occurred. The animal protocols used in this study were approved by the Beijing University of Traditional Chinese Medicine's Institutional Animal Care and Uses Committee.

Plasma collection and preparation

Prior to experiments, the rats were fasted for 12 h but supplied with drinking water. Before drug administration, blank blood samples, taken from the orbit, were injected into heparinized Eppendorf tubes. Next, YHSP was orally administered to the rats in a dose of 1.5 g/kg (clinical equivalent dose) [7] and 1 mL/100 g (dose volume/body weight). Medicated blood samples were collected at time points corresponding to 0.5, 1, 2, 4, 8, 12 and 24 h after dosing in an identical method to the blank specimens. All samples were immediately centrifuged at 4,000 rpm for 10 min at 4 °C. The plasma samples were transferred to Eppendorf tubes and stored at −80 °C. For final analysis, the medicated plasma samples of each rat were mixed, including all time points. A total of six rat plasma samples were prepared. One-hundred µL of plasma was treated with 300 µL acetonitrile to precipitate the protein; samples were then vortex mixed for 3 min and centrifuged at 12,000 rpm for 10 min. The suspension was then transferred to a new tube and dried by nitrogen in a water bath at 40 °C. The residue was re-dissolved with 100 µL methanol and centrifuged at 12,000 rpm for 10 min for further analysis.

UPLC-Q-TOF/MS analysis

Analysis of the chemical composition of the YHSP decoction was carried out using high-resolution liquid chromatography tandem MS.

UPLC conditions. Chromatographic separation was achieved using the ACQUITY UPLC system (Waters Corporation, Milford, MA, USA). The chromatographic column used was a Waters Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm). The mobile phase consisted of: (A) 0.1 % formic acid aqueous solution and (B) acetonitrile, while the gradient elution was optimized as follows: 0–2 min, 2%–20% B, 2–7 min, 20%–40% B, 7–10 min, 40%–60% B, 10–16 min, 60%–98% B, 16–17 min, 98%–2% B and 17–18 min, 2% B. A flow rate of 0.3 mL/min and a column temperature of 35 °C were set for separation. The injection volume was 5 µL.

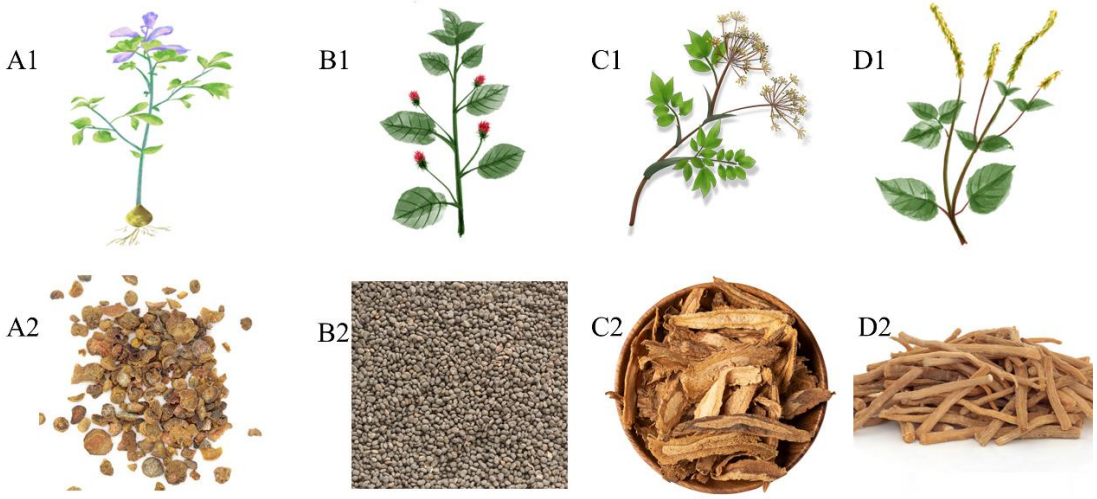


Figure 1 The figure about the whole plant and medicinal sites of four herbs of YHSP. A1, the plant of *Corydalis yanhusuo W. T. Wang*; A2, the medicinal sites of *Corydalis yanhusuo W. T. Wang*; B1, the plant of *Psoralea corylifolia L*; B2, the medicinal sites of *Psoralea corylifolia L*; C1, the plant of *Angelica sinensis* (*Oliv.*) *Diels*; C2, the medicinal sites of *Angelica sinensis* (*Oliv.*) *Diels*; D1, the plant of *Achyranthes bidentata Bl.*; D2, the medicinal sites of *Achyranthes bidentata Bl*. YHSP, Yanhusuo powder.

doi: 10.12032/TMR20210603232

MS conditions. Mass spectra were acquired on a SYNAPT G2-Si HRMS (Waters Corporation, Milford, MA, USA) equipped with an electrospray ionization (ESI) source. MS data were collected from m/z 50–1500 Da in positive MS^E continuum modes. ESI conditions were set as follows: capillary voltage, 3.0 kV; cone voltage, 40 V; source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 600 L/h; cone gas flow, 50 L/h; high energy channel collision voltage, 10–75 V and ion acquisition rate, 0.2 s. To ensure mass accuracy and reproducibility, leucine encephalin (m/z 556.2771 in positive mode) was used to lock the mass at a concentration of 1 μg/mL and the flow rate at 10 μL/min. Data acquisition and processing were performed using MassLynx V4.1 and UNIFI Scientific Information System V1.7 (Waters Corporation, Milford, MA, USA), respectively.

UPLC-MS/MS analysis

UPLC conditions. The analysis was performed through a similar ACQUITY UPLC system (Waters Corporation, Milford, MA, USA). The liquid phase parameter settings, such as the chromatographic column, mobile phase, gradient elution procedures and column temperature, were consistent with the above method in "Q-TOF methods".

MS conditions. Mass spectra were acquired on a XEVO TQS micro (Waters Corporation, Milford, MA, USA) equipped with an ESI source operated in positive ESI mode. ESI conditions were as follows: desolvation gas flow, 650 L/min; desolvation temperature, 350 °C and source temperature, 120 °C. Analytes were qualitatively measured using multiple-reaction monitoring (MRM) mode. Cone voltage, capillary voltage and collision energy values were optimized to obtain the highest response value. All data acquisition and analysis were controlled by Waters MassLynx V4.2 software.

Network Pharmacology

Target genes of YHSP. Molecular files of the absorbed constituents in YHSP were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/) or drawn by ChemDraw. Then, the targets of the constituents were collected from SwissTargetPrediction (http://www.swisstargetprediction.ch/).

OA target genes. We used "osteoarthritis" as the key word to gather information regarding OA targets from the following sources: Online Mendelian Inheritance in Man (https://www.omim.org/) and GeneCards (http://www.genecards.org/). The Draw Venn Diagrams website (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to map the intersection between targets of the absorbed components and genes associated with OA, in order to obtain the relevant direct targets.

Protein-protein interaction (PPI) construction and topological analysis. All targets were imported into String database (https://string-db.org/) for PPI data analysis with the organism set to *Homo sapiens*. Afterwards, the TSV file obtained from the String website was transferred to Cytoscape software (V3.7.1). Using Cytoscape software, we conducted a topological analysis of each node in the interactive network based on three parameters, specifically "degree", "betweenness centrality" and "closeness centrality" [13].

Enrichment analysis. Gene Ontology enrichment analysis and Kyoto Gene and Genome Encyclopedia (KEGG) pathway enrichment analysis were realized using the DAVID (https://david.ncifcrf.gov/) website. Relevant results of the analysis were screened according to the false discovery rate < 0.05.

Construction of networks. To visualize the interrelationships among constituents and potential targets, Cytoscape 3.7.1 was applied for compound-target-disease network construction [14].

Molecular docking simulation

Molecular docking was used to further verify the credibility of the main targets in the related signaling pathways; this verification process was completed with the help of AutoDock 4.2.6 software. The structure of the main active ingredient was downloaded from the PubChem website, while the Protein Data Bank platform (http://www.rcsb.org/pdb/home/home.do) was used to download the protein structure of the main target in the relevant signaling pathway. The binding energy (△Gbind) and inhibition constant (Ki) were used as reference indicators, while glucosamine sulfate, artesunate and shikonin were used as controls to obtain the binding of 20 ligand compounds to PI3K-AKT target proteins in the chondrocyte apoptosis-related pathway; the lower the △Gbind and Ki, the stronger the combination [15].

Results

A three-step strategy for screening and identifying prototypes in plasma samples

Systematic identification of TCM compounds in a complex biological sample is typically carried out using UPLC-Q-TOF/MS [16]. This technique provides fast scanning, high-mass resolution, as well as high throughput capabilities. However, for compounds at trace levels in biological samples, UPLC-Q-TOF/MS is limited by its sensitivity; under these circumstances, this method will induce peak loss or fail to detect compounds, thus affecting the accuracy of the results. On the contrary, UPLC-MS/MS has advantages such as high sensitivity, robustness and reproducibility for biopharmaceutical analysis [17]. As depicted in Figure 2, in this study, a more sensitive and accurate three-step strategy based on the two kinds of UPLC-MS techniques for identifying prototype

components in rat plasma after oral administration of YHSP was established.

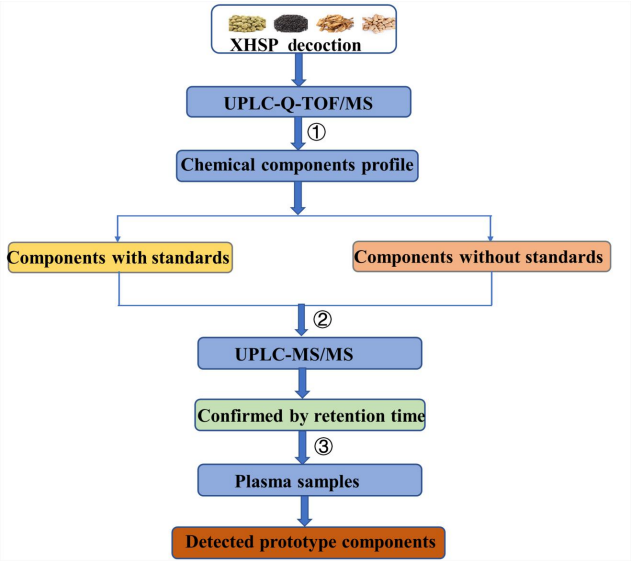


Figure 2 A general flowchart illustrating the application of UPLC-Q-TOF/MS combined with UPLC-MS/MS to characterization of the prototype components in vivo of TCM: YHSP as a case study. TCM, traditional Chinese medicine; YHSP, Yanhusuo powder; UPLC-Q-TOF/MS, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography-triple quadrupole mass spectrometry.

Firstly, the chemical component profile of YHSP was rapidly identified using UPLC-Q-TOF/MS. Secondly, an UPLC-MS/MS method was established to verify the components obtained by UPLC-Q-TOF/MS. The same liquid chromatography method was used to avoid systematic error. The compounds with standard substances were selected to verify the accuracy of the UPLC-MS/MS method based on the consistencies of their retention times. The compounds without standard substances were further confirmed according to retention time and UPLC-Q-TOF/MS fragments. Compounds with high retention time consistency were selected as compounds with high reliability for biopharmaceutical analysis. Thirdly, the UPLC-MS method was employed to profile the absorbed components of YHSP in plasma samples.

Preliminary identification of the component profile of YHSP by UPLC-Q-TOF/MS

UPLC-Q-TOF/MS was applied in order to conduct a preliminarily component profile of YHSP with positive ionization MS^E mode. UNIFI software was employed for rapid chemical composition identification. A total of 34 components were identified, including 14 alkaloids, 7 flavonoids, 4 coumarins and 9 other components. The identification process was performed mainly through comparison with reference compounds and the analysis of molecular formulas, fragment ions, and chemical structure information obtained from the literature and public databases. A summary of the identified compounds following UPLC-Q-TOF/MS analysis is presented in Figure 3 and (Supplementary Table S1).

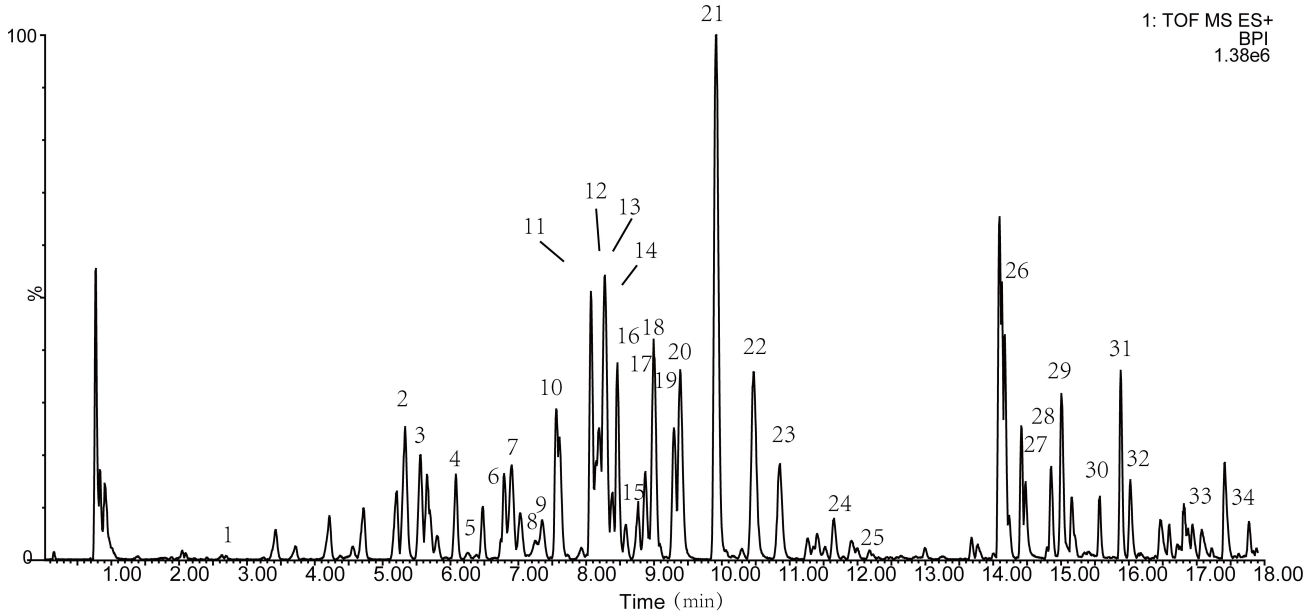


Figure 3 The base peak intensity chromatography of YHSP decoction using UPLC-Q-TOF/MS. YHSP, Yanhusuo powder; UPLC-Q-TOF/MS, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry.

doi: 10.12032/TMR20210603232

Further confirmation of the components in YHSP by UPLC-MS/MS

The components identified by UPLC-Q-TOF/MS were unambiguously or tentatively characterized. Compared with UPLC-MS/MS, UPLC-Q-TOF/MS has a lower sensitivity, thus making low content substances difficult to detect, especially in biological samples. On account of this, in the present study, an UPLC-MS/MS method was applied for further exploration and verification of the components of YHSP for the purpose of facilitating the detection of low content absorbed components. In order to ensure the accuracy of this method, the components with standards of YHSP were selected to establish the UPLC-MS/MS screening method. The applicability of the method was confirmed by comparing the retention times of the standards on the two instruments. It turns out that the retention time of the standards, including ferulic acid, ecdysterone, columbamine, tetrahydropalmatine, psoralen, angelicin, neobavaisoflavone, psoralidin and bakuchiol, were similar following UPLC-Q-TOF/MS and UPLC-MS/MS analysis, with differences of less than 0.2 s. Next, the same strategy was employed to screen the remaining components, identified by UPLC-Q-TOF/MS, that did not have standards. With the exception of 2.54 min for senkyunolide A; 6.18 min for tetrahydrocolumbamine and 8.58 min for senkyunolide H or I, the components were all found to have similar retention times under UPLC-Q-TOF/MS analysis by adjusting the capillary voltage, collision energy and cone voltage. In total, 30 components (including 5 compound isomers) were further confirmed via UPLC-MS/MS; see Figure 4 for details.

Identification of the absorbed components of YHSP in rat plasma based on UPLC-MS/MS

In this study, the absorbable bioactive constituents of YHSP in rat plasma were analyzed. The UPLC-MS/MS method, established above, was performed to screen the constituents. The absorbed constituents are shown in Table 1. A total of 20 prototype constituents were found in the dosed plasma, however, only five of these constituents could be identified using UPLC-Q-TOF/MS, as shown in Figure 3. This indicated that the UPLC-MS/MS method has a higher sensitivity, which can enrich the components to be tested in a more targeted way, especially during in vivo TCM compound analysis.

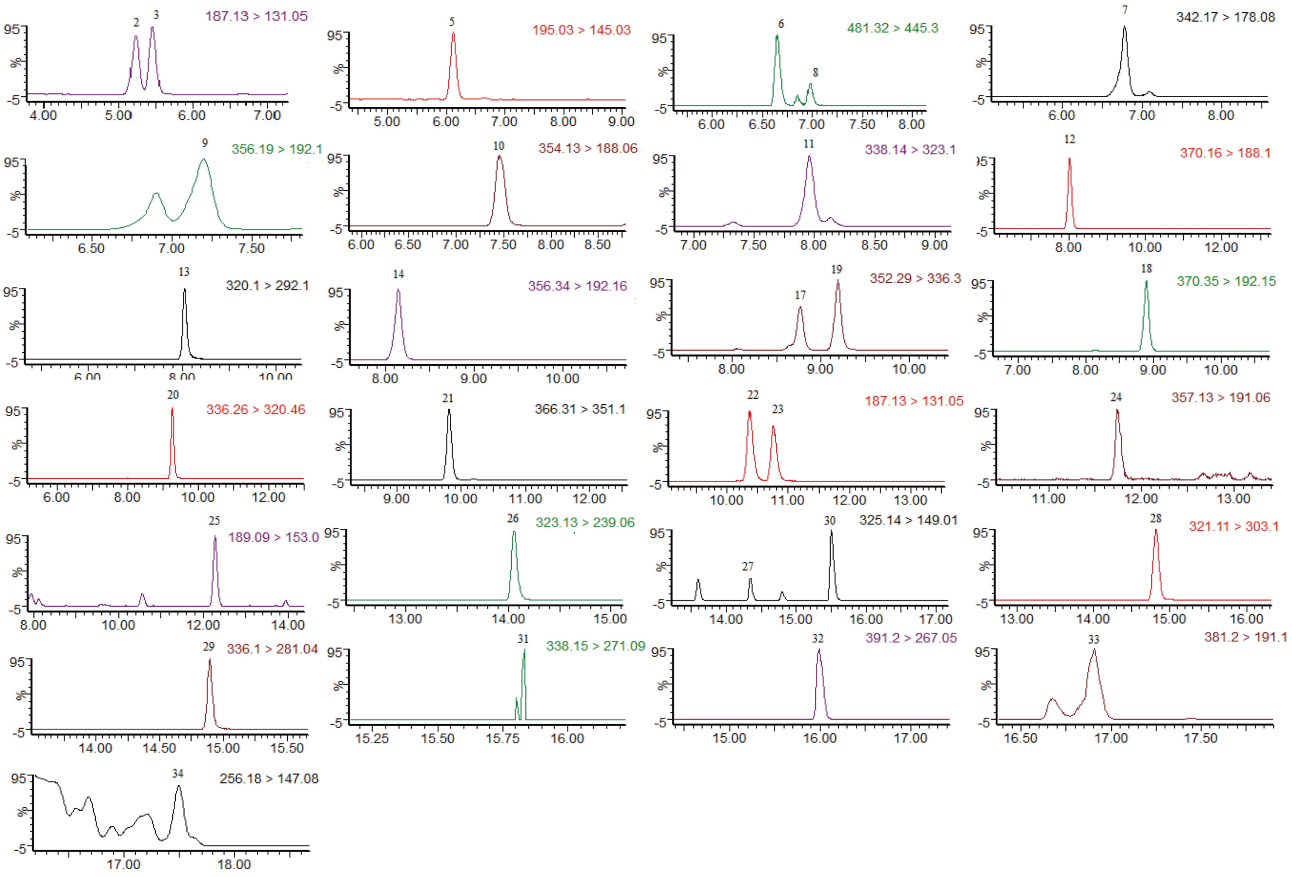


Figure 4 MRM ion chromatography of YHSP decoction using UPLC-MS/MS. MRM, multiple-reaction monitoring; YHSP, Yanhusuo powder; UPLC-MS/MS, ultra-performance liquid chromatography-triple quadrupole mass spectrometry.

Table1 Identification of components profile of YHSP by UPLC-MS/MS

No.	t_R (min)	Compounds	Formula	MRM transition precursor ion > product ion	Cone voltage	Collision energy	Ion mode	Source
1	6.80	Tetrahydrojatrorrhizine	$C_{20}H_{23}NO_4$	342.17 > 178.08 342.17 > 163.05	4 4	20 20	+ +	YHS
2	7.47	Protopine	$C_{20}H_{19}NO_5$	354.13 > 190.54 354.13 > 188.06	4 2	32 28	+ +	YHS
3	7.96	Columbamine	$C_{20}H_{20}NO_4$	338.14 > 323.10 338.14 > 308.08	4 4	20 20	+ +	YHS
4	8.27	Allocryptopine	$C_{21}H_{23}NO_5$	370.14 > 188.10 370.14 > 338.13	4 4	20 20	+ +	YHS
5	8.28	Tetrahydropalmatine	$C_{21}H_{25}NO_4$	356.34 > 192.16 356.34 > 148.27	6 6	30 60	+ +	YHS
6	8.78	Dehydrocorybulbine	$C_{21}H_{22}NO_4$	352.29 > 336.30 352.29 > 320.50	4 2	20 32	+ +	YHS
7	8.91	Corydaline	$C_{22}H_{27}NO_4$	370.35 > 192.15 370.35 > 188.25	6 6	30 22	+ +	YHS
8	9.20	Palmatine	$C_{21}H_{22}NO_4$	352.29 > 336.30 352.29 > 308.12	2 4	32 20	+ +	YHS
9	9.30	Berberine	$C_{20}H_{18}NO_4$	336.26 > 320.46	4	26	+	YHS
10	9.82	Dehydrocorydaline	$C_{22}H_{24}NO_4$	366.31 > 351.10 366.31 > 308.82	2 2	20 42	+ +	YHS
11	10.35	Psoralen	$C_{20}H_{16}O_5$	187.13 > 131.05	24	22	+	BGZ
12	10.74	Angelicin	$C_{20}H_{16}O_5$	187.13 > 131.05	24	22	+	BGZ
13	12.29	Butylidenephalide	$C_{12}H_{12}O_2$	189.09 > 153.06 189.09 > 143.08	4 4	25 25	+ +	DG
14	14.05	Neobavaisoflavone	$C_{20}H_{18}O_4$	323.13 > 239.06	4	25	+	BGZ
15	14.34	Bavachin	$C_{20}H_{20}O_4$	325.14 > 149.01 325.14 > 269.06	4 4	30 30	+ +	BGZ
16	14.79	Corylin	$C_{20}H_{16}O_4$	321.11 > 303.10	4	25	+	BGZ
17	14.91	Psoralidin	$C_{20}H_{16}O_5$	336.10 > 281.04 336.10 > 308.21	4 4	20 20	+ +	BGZ
18	15.50	Corylifolinin	$C_{20}H_{20}O_4$	325.14 > 149.01 325.14 > 269.06	4 4	30 30	+ +	BGZ
19	16.01	Corylifol A	$C_{25}H_{26}O_4$	391.20 > 267.05 391.20 > 239.06	4 4	30 30	+ +	BGZ
20	16.90	Levistolide A	$C_{24}H_{28}O_4$	381.20 > 191.10 381.20 > 335.20	4 4	25 25	+ +	DG

MRM, multiple-reaction monitoring; YHSP, Yanhusuo powder; UPLC-MS/MS, ultra-performance liquid chromatography-triple quadrupole mass spectrometry; YHS, *Corydalis yanhusuo W. T. Wang*; BGZ, *Psoralea corylifolia L*; DG, *Angelica sinensis* (*Oliv.*) *Diels*.

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Mechanism of YHSP in treating OA by network biology strategy analysis

Potential targets of the absorbed constituents of YHSP. The 20 absorbed components obtained above were subjected to further network biology strategy analysis, with potential targets gathered from SwissTargetPrediction. In total, 630 targets were revealed, corresponding to 20 absorbed constituents in YHSP after the removal of repeated targets.

Potential therapeutic targets and pathways. By searching the databases of Online Mendelian Inheritance in Man and GeneCards, a total of 979 disease targets were obtained. One-hundred and thirty-one direct targets related to the effects of YHSP in treating OA were obtained through the interaction of component targets and disease targets (Supplementary Figure S1). String database [18] is a database containing known and predicted large-scale PPI. In this study, network topology analysis was performed by String database. As can be seen from Supplementary Figure S2 and S3, targets such as AKT1, TNF, CXCL8, signal transducer and activator of transcription 3 and mitogen-activated protein kinase 8 (MAPK8) play an important role in the network. Gene Ontology analysis results (Supplementary Figure S4) indicted that the relevant biological processes mainly involved inflammatory responses and vascular protection. Additionally, 29 KEGG pathways were obtained from KEGG analysis (Supplementary Figure S5) ($P < 0.05$), the results of which can be divided into five aspects: PI3K-AKT, MAPK, apoptosis, Ras, vascular endothelial growth factor and ErbB are 6 pathways related to cell proliferation, apoptosis and differentiation; chemokine, TNF and NF-κB are 3 pathways associated with inflammation; B cell receptor, T cell receptor and Toll-like receptor are 3 immune pathways and forkhead box O and adenosine monophosphate-activated protein kinase are 2 oxidative stress pathways. KEGG results indicate that YHSP might be effective in treating OA through multiple pathways and multiple targets.

Compound-target-pathway network construction. To more intuitively represent the relationships among compounds, targets and pathways, a complicated compound-target-pathway network was constructed using Cytoscape 3.7.1 (Figure 5). The complex connection between components, targets and pathways in the network diagram visually shows the complex mechanism of YHSP in the treatment of OA.

Docking stimulation verification

In this study, we tested the binding ability between the main active ingredients of YHSP and the core target through molecular simulation docking and, further, screened the active ingredients. According to the predicted results of the network biology strategy and the pathological changes associated with OA, we selected the target of YHSP and anti-chondrocyte apoptosis and the PI3K-AKT signaling pathway for molecular docking verification. By searching the related literature, we determined that the receptor protein molecules are caspase-3, Bax, Bcl-2, AKT, PI3K and mammalian target of rapamycin (mTOR).

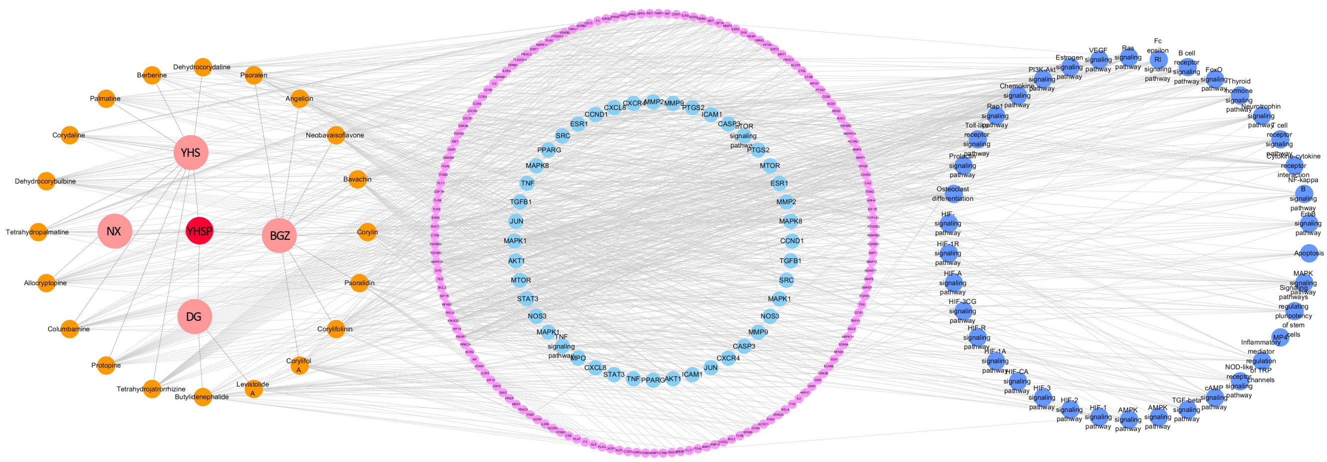

Figure 5 The interaction network of TCM-components-targets-pathways network. YHSP is represented by the red node, the 4 herbs are represented by the pink nodes, orange nodes represent 20 prototype components; azure nodes represent 21 core targets, and rose nodes represent a target other than the core target; blue nodes represent pathways; the interactions between nodes are linked by edges. TCM, traditional Chinese medicine; YHSP, Yanhusuo powder; YHS, *Corydalis yanhusuo W. T. Wang*; BGZ, *Psoralea corylifolia L*; DG, *Angelica sinensis (Oliv.) Diels*; NX, *Achyranthes bidentata Bl*.

Glucosamine sulfate is a drug commonly used in the clinical treatment of OA, therefore, this drug was selected as a positive drug in terms of efficacy in this trial. According to the molecular docking experiments, the average values of △Gbind and Ki of the positive control compound, glucosamine sulfate, bound to these six proteins were −0.85 kcal/mol and 74.88 μmol/L, respectively. Artesunate and shikonin are compounds reported in the literature to have a regulatory effect on the expression of the six proteins in the PI3K-AKT pathway, therefore, these two compounds were selected as positive drugs for the pathway. The experimental results show that the mean values of △Gbind and Ki of artesunate were −3.04 kcal/mol and 41.66 μmol/L, respectively. The mean values of △Gbind and Ki of shikonin were −3.03 kcal/mol and 35.29 μmol/L, respectively. The combination of these three positive drugs was used as a reference to evaluate the combination of 20 blood prototype components. The results (Supplementary Table S2) show that the average binding energies of these 20 components are all less than the three positive control compounds, indicating that the 20 blood-infused components and the six ligand proteins all have potential binding capabilities, and the binding can match each other spatially to achieve a firm and stable binding. The docking results of the compounds with the best binding ability to these six receptor proteins, sorted according to △Gbind values from lowest to highest, are shown in Figure 6.

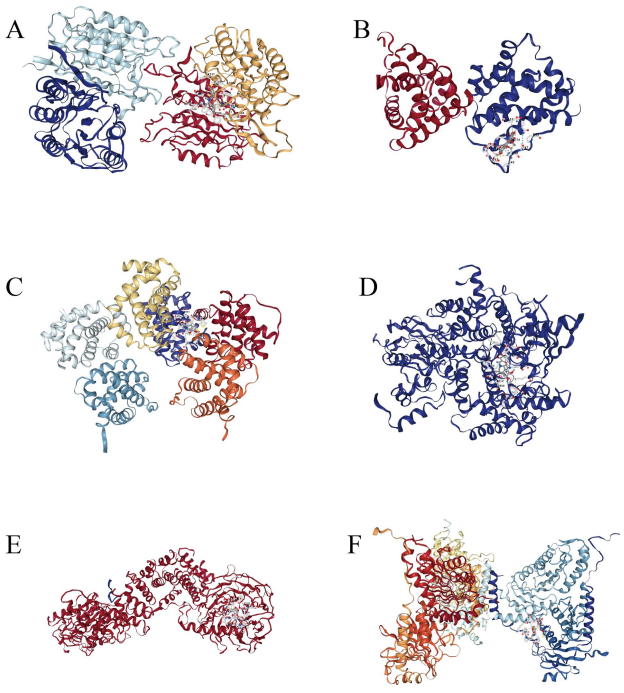

Figure 6 Compounds with the best binding ability to these six receptor proteins. A, protopine and caspase-3; B, dehydrocorybulbine and Bax; C, dehydrocorybulbine and Bcl-2; D, corydaline and PI3K; E, protopine and AKT1; F, protopine and mTOR. mTOR, mammalian target of rapamycin.

Discussion

YHSP is composed of *Corydalis yanhusuo*, *Psoralea corylifolia*, *Achyranthes bidentata* and *Angelica sinensis*. According to the theory of TCM, YHSP has effects of tonifying the kidney, strengthening bone, promoting blood circulation and relieving pain, producing good clinical effects in the treatment of OA. In the current study, a novel analytical method combining the use of UPLC-Q-TOF/MS and UPLC-MS/MS was used to identify multiple components in YHSP from rat plasma. The combination of these two sets of MS not only avoids the failure to detect components caused by the low sensitivity of UPLC-Q-TOF/MS, but also makes full use of the advantages of the high sensitivity of UPLC-MS/MS, creating a more comprehensive and accurate determination of the blood components of YHSP. By comparing the retention times and MS data of standard samples and data in the literature, 20 types of prototype compounds were successfully isolated and identified, including alkaloids, flavonoids and coumarins, of which psoralen, angelicin, tetrahydropalmatine, corydaline, protopine and berberine exerted higher absorption areas. The UPLC-MS strategy established in our study is more credible and improves the sensitivity of the analysis of absorbed prototype components of TCM in biological samples.

Studies have shown that OA is a degenerative osteoarticular disease characterized by chronic synovitis, degeneration of articular cartilage and remodeling of subchondral bone [19]. The pathological changes of OA mainly involve degradation of the extracellular matrix of articular chondrocytes, apoptosis of chondrocytes, and autoimmune responses [20]. Network biology strategy studies showed that the 20 components are closely related to multiple pathways, including cell proliferation, apoptosis, inflammatory responses, immune regulation, oxidative stress and bone metabolism regulation, fully embodying the multi-pathway and multi-target effects of YHSP in treating OA. Compared with previous research results, the results of this study not only include the previous important results, but also refine the specific pathways of YHSP in the treatment of OA; this is especially true regarding the inflammatory and apoptotic pathways, such as PI3K-AKT and MAPK, indicating that the current prediction of mechanisms according to the blood ingredients is more comprehensive and accurate, as well as more convenient for subsequent experimental verification. It has been demonstrated that inflammation, together with chondrocyte apoptosis, plays a necessary role in the progression of OA [21, 22]. It's worth noting that,

in this study, both the apoptotic pathway and inflammatory pathway occupied prominent positions in the KEGG results. Therefore, in our follow-up molecular docking studies, we chose to validate the blood ingredients and apoptosis and the main inflammatory pathway, PI3K-AKT. The PI3K-AKT signaling pathway is a major antiapoptotic pathway that is important for cell proliferation, differentiation and apoptosis. The PI3K-AKT pathway is also one of the most significant pathways regulating chondrocyte proliferation, apoptosis and matrix remodeling [23]. Akt is a principal effector in the PI3K-Akt signaling pathway. Furthermore, Akt has the function of directly activating mTOR, which is a highly conserved serine/threonine kinase that works as a major negative regulator of autophagy, affecting chondrocyte metabolism as well as the progression of OA. mTOR gene knockout can improve the expression of autophagy signals, as well as inhibit the degeneration of articular cartilage and the degree of apoptosis [24, 25]. In this study, Akt was the core protein with a three-degree ranking. Eight ingredients, such as tetrahydropalmatine, protopine and corylifolinin, interacted with Akt, indicating that the effects of YHSP are probably connected to the PI3K/AKT/mTOR pathway regulated by chondrocyte apoptosis and chondrocyte autophagy.

MAPK is another important pathway leading to the apoptosis of chondrocytes that can also activate downstream protein kinases or transcription regulators, such as Bcl-2 and Sox9, and further regulate cell activity [26]. An increasing number of studies have demonstrated that inflammatory factors, such as interleukin-1 and TNF-α, can activate the MAPK signaling pathway, causing increased expression of matrix metalloproteinases in synovial fluid, resulting in a series of reactions including chondrocyte apoptosis and cartilage destruction in OA [27]. The network biology strategy analysis indicated that nine absorbed ingredients of YHSP, including psoralidin, palmatine and dehydrocorybulbine, were closely related to the MAPK pathway. Therefore, we can speculate that MAPK is another important pathway of YHSP.

In this experiment, molecular docking techniques were used to simulate the interactions between 20 blood-entering components and the potential efficacy targets of YHSP. According to the results of this study, it can be preliminarily judged that protopine, dehydrocorybulbine, angelicin, tetrahydropalmatine, psoralen, berberine and corydaline are seven compounds with good virtual binding activity with caspase-3, Bax, Bcl-2, AKT, PI3K, mTOR and other proteins, which may be the potential pharmacodynamic basis of YHSP. The mechanism of YHSP in treating OA may be that the chemical component interacts with proteins in the PI3K-AKT pathway, resulting in changes in protein expression, thus improving chondrocyte apoptosis. Follow-up experiments must be conducted to further verify these results via cellular experiments.

Conclusion

In this paper, an integrated strategy based on UPLC-Q-TOF/MS combined with UPLC-MS/MS was developed for identifying the prototype constituents in rat following the oral administration of YHSP. A total of 20 compounds were initially identified in rat plasma. The major absorbed compounds included tetrahydropalmatine, angelicin and psoralen. Additionally, the pharmacological mechanism of YHSP was further investigated by the network biology strategy and molecular docking stimulations based on the absorbed compounds. The results show that the therapeutic effect of YHSP on OA may be via 131 biological targets related to multiple pathways, including cell proliferation, apoptosis, inflammatory responses, immune regulation, oxidative stress and bone metabolism regulation. The main signaling pathways involved were PI3K-Akt, MAPK, interleukin-17 and TNF-β. In summary, the results of this study illustrate that OA treatment using YHSP probably acts through anti-inflammatory effects, anti-apoptotic effects on chondrocytes, inhibition of extracellular matrix degradation, immune regulation and oxidative stress pathway regulation. Our results highly coincide with the integrity principle of TCM theory, which may open up a new strategy in the study of TCM in the future. Furthermore, the bioactive ingredients, biological targets and signaling pathways predicted by the network biology strategy approach will be confirmed and validated using animal models of OA and cell models of chondrocyte apoptosis in our further studies.

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