# Therapeutic Effects of Jiedu Huoxue Decoction in Chronic Prostatitis/Chronic Pelvic Pain Syndrome: Network Pharmacology, Molecular Docking and Experimental Evidence

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**Research Article**

**Keywords:** Jiedu Huoxue Decoction, Chronic Prostatitis/Chronic Pelvic Pain Syndrome, Network Pharmacology, Molecular Docking, Experimental Evidence

**Posted Date:** March 29th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1424400/v1

# Abstract

**Background:** Jiedu Huoxue decoction (JDHXD) can be effectively alleviated chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) patients' pelvic pain, lower urinary tract symptoms, and other discomforts. But its mechanism of action involving multi-component, multi-target and multi-pathway is unclear.

**Objectives:** Integrated strategy of network pharmacological prediction, molecular docking, and experimental validation elucidate potential mechanism of JDHXD in treating CP/CPPS.

**Study design and methods:** Two traditional Chinese medicine systems pharmacology databases and three disease related databases were applied to identify the herbs active components and the putative targets of CP/CPPS. Using the Cytoscape to constructed and analysis the herbs-compounds-targets network. Subsequently, the protein-protein interaction (PPI) was investigated by STRING database. Using DAVID databases to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The binding of core active compounds with hub targets were assessed by molecular docking. Furthermore, in vivo efficacy of JDHXD was validated in CP/CPPS rat model. The key signal pathway of network pharmacological prediction would be validated by animal experiment.

**Result:** In total, 149 active compounds of 10 Chinese herbals and 909 potential CP/CPPS-related targets were identified. Network pharmacology revealed 161 common targets between 136 potential active compounds and CP/CPPS. After topology analysis, 5 core active compounds and 18 hub targets have been screened. PPI analysis obtained 18 hub targets. Based on enrichment analysis of 161 targets, we obtained GO function 277 items and KEGG pathway 114 items. Molecular docking verification shown that the top 5 hub targets were well binding with 5 core active compounds. Animal experiment shown that JDHXD had protective effect on CP/CPPS. JDHXD can significantly down-regulated the key gene expression (JNK, ERK1/2 and P38) and inhibit phosphorylation of ERK1/2、JNK and P38 in the MAPK signaling pathway.

**Conclusions:** This study demonstrated potential mechanism of JDHXD in treating CP/CPPS. Network pharmacological, molecular docking and experimental verification revealed its multi-component, multi-target and multi-pathway treatment characteristics. The reliability and effectiveness of those method are very helpful to explore the material basis and mechanism of traditional Chinese medicine.


# Introduction

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), also known as category Ⅲ prostatitis, is defined as chronic pelvic pain not caused by other identifiable pathology in light of the National Institutes of Health (NIH) [1]. The morbidity of CP/CPPS is about 2.2% ~16.0% in 20~40 years men worldwide, and in Chinese males is approximately 8.4% [2,3]. As a common, chronic and recurrent urologic condition, CP/CPPS has a variety of clinical symptoms, such as pelvic pain or perineal pain, irritative or obstructive

voiding symptoms, sexual dysfunction or psychological disorders. And these clinical symptoms effect patient's quality of life, and brings about a variety of physical and psychological problems. What's more, the etiology and pathogenesis of CP/CPPS are complex and unclear, and it is still one of the refractory diseases. Current studies have shown that the pathogenesis of chronic prostatitis is mainly related to pathogen infection, oxidative stress, cytokines and collective autoimmunity, genetic factors and other factors [4]. Therefore, in order to alleviate the symptoms and pain of patients, multiple treatments have been used clinically, such as antibiotics, alpha-blockers, non-steroidal anti-inflammatory analgesics, etc. However, due to the lack of specificity in these treatments, it is difficult to achieve satisfactory comprehensive efficacy, and the final evolution of CP/CPPS is prone to recurrent attacks and prolonged course of disease. Therefore, it is greatly significance to search drugs to treat chronic prostatitis and reveal its mechanism of action.

Traditional Chinese medicine (TCM) has obvious advantages in treating CP/CPPS based on syndrome differentiation, gaining more attention in the treatment of CP/CPPS due to its low adverse effects, high efficacy potency than other traditional therapies. Based on his long-term clinical practice experiences and combined with the etiology and pathogenesis of CP/CPPS, Professor Wenqiu Yu, prestigious TCM doctor, developed the herbal formula of Jiedu Huoxue decoction (JDHXD) to treat CP/CPPS. This herbal formula is made up of ten Chinese herbs (Table 1). Our previous studies have revealed that after JDHXD treatment, patients' pelvic pain, lower urinary tract symptoms, and other discomforts can be alleviated effectively [5]. And the pharmacological experiments showed that JDHXD can eliminate the inflammatory response through reducing the levels of pro-inflammatory cytokines TNF-α and IL-8, and increasing the expression of anti-inflammatory cytokines IL-10 in estradiol benzoate (EB)-induced CP/CPPS rats [6,7]. Further research found that NF-κB superfamily is involved in EB-induced rat CP/CPPS model, and JDHXD can alleviate prostate injure at least partly by suppressing the activation of NF-κB signal pathway [8]. Moreover, We obtained the miRNA expression profile of EB-induced CP/CPPS rats through miRNA high-throughput chip technology in the early stage, and the results demonstrated that JDHXD had an intervention effect on the differential miRNAs by regulating cell proliferation, autophagy, immune regulation and other important biological functions [9].

However, JDHXD has many herbs, rich ingredients and extremely complicated synergistic effects, and the underlying molecular mechanism is not yet fully elucidated. Laboratory studies of single molecule or single pathway cannot reveal the full extent of the molecular mechanisms responsible for their efficacy, which would limit development and utilization of JDHXD. Network pharmacology have shown particular utility in analyzing multi-component, multi-target and multi-pathway to reveal the underlying therapeutic mechanisms of herbal formulae at the molecular level. Based on systems biology, molecular biology and pharmacology, network pharmacology combines online databases and software to mine and analyze and display the intersection genes of drugs and diseases [10]. The visualized data can more directly explain the correlation between TCM compounds and diseases, and comprehensively explore the possible mechanism of prescription in treating diseases [11,12].

Therefore, this study used network pharmacology method to systematically identify active compounds present in JDHXD and their plausible corresponding targets, analyze the signaling pathways and biological process networks regulated by the product and evaluating its effects on disease-associated gene sets or networks, and culminates with the investigation of the mechanism of JDHXD in the treatment of CP/CPPS. Finally, the key signal pathway of network pharmacological prediction would be validated by molecular docking and animal experiment.

**Table 1. The information of Chinese Herbal Medicine in JDHXD.**

| Herb Name | Latin name | Family | Genus | Species | Part or organ |
|---|---|---|---|---|---|
| Tufuling | SMILACIS GLABRAE RHIZOMA | Liliaceae | Smilacaceae | glabra Roxb | Rhizome |
| Huzhang | POLYGONI CUSPIDATI RHIZOMA ET RADIX | Polygonaceae | Polygonum | cuspidatum | Rhizome and root |
| Fenbixie | DIOSCORE AEHYPOGLAUCAE RHIZOMA | Dioscoreaceae | Dioscorea | hypoglauca Palibin | Rhizome |
| Cheqianzi | PLANTAGINIS SEMEN | Plantaginaceae | Plantago | depressa Willd/asiatica L. | Seeds |
| Wangbu Liuxing | VACCARIAE SEMEN | Caryophyllaceae | Vaccaria | segetalis （Neck.） Garcke | Seeds |
| Ezhu | CURCUMAE RHIZOMA | Zingiberaceae | Curcuma | phaeocaulis VaL. | Rhizome |
| Chuanniuxi | CYATHULAE RADIX | Amaranthaceae | Cyathula | officinalis Kuan | Root |
| Yuanzhi | POLYGALAE RADIX | Polygalaceae | Polygala | tenuifolia Willd./sibirica L. | Root |
| Shichangpu | ACORI TATARINOWII RHIZOMA | Araceae | Acorus | tatarinowii Schott | Rhizome |
| Gancao | GLYCYRRHIZAE RADIX ET RHIZOMA | Leguminosae | Glycyrrhiza | uralensis Fisch./inflata Bat./glabra L. | Rhizome and root |

Reference: Pharmacopoeia of the People's Republic of China (2020 edition)

# Materials And Methods

### Network pharmacology Prediction

## Active compounds Collection and Active Target Identification

In order to screen compounds ingredients of JDHXD, we searched all the herbs in the two databases (Traditional Chinese Medicine Database and Analysis Platform, TCMSP, https://tcmsp-e.com/; The Encyclopedia of Traditional Chinese Medicine, ETCM,http://www.tcmip.cn/ETCM/index.php) [13,14]. And we screened compounds by oral bioavailability (OB) value ≥30% and drug likeness (DL)≥0.18 as active compounds. Moreover, according to the content determination from Pharmacopoeia of the People's Republic of China (2020 Edition), We included principal components of the herbs that OB or DLfailedto meet the established conditions[15].

The potential targets of active compounds were collected from TCMSP and ETCM database. Next, we checked all the corresponding targets and translated to gene name via UniProt database (https://www.uniprot.org/)[16]. Duplicated, nonhuman, and nonstandard targets were eliminated.

## CP/CPPS related targets collection

To assemble potential targets associated with CP/CPPS, we searched the DisGeNET database (https://www.disgenet.org/home/), Online Mendelian Inheritance in Man (OMIM,https://omim.org/) and The Human Gene Database (GeneCards,https://www.genecards.org/) databases[17–19]. We used "chronic prostatitis" and" chronic pelvic pain syndrome" as keyword searching three databases respectively. And after merging, we deleted duplicates to establish a CP/CPPS related targets dataset. Targets were normalized to the official gene symbols using UniProt database.

## Herbs-compounds-targets-disease network construction and analysis

In order to illuminate and visualize the relationship between the active compounds and the targets, we constructed the herbs-compounds-targets-disease network by means of Cytoscape□version3.8.2, https://cytoscape.org□[20]. After collating drug and disease targets, these two target datasets were compared and filtered to identify common targets, which were defined as the targets of JDHXD in treating CP/CPPS. Then, those common targets, active compounds, herbs□and disease were input into Cytoscape software to construct herbs-compounds-targets-disease network. Next, we utilized Network Analyzer, a Cytoscape plugin, to perform topology analysis on nodes. We set 2-fold of the median degree as screening criteria to evaluate the essentiality of each node within the entire network and to confirm core active compounds.

## Protein-protein interaction (PPI)analysis

The STRING database (Version11.5,https://string-db.org/) was used to investigate interaction and multi-targeted regulations of common targets[21]. we imported gene symbols of common targets into STRING database. The species was set as "Homo sapiens" and Confidence score more than 0.4 is defined as significant. Finally, the result was visualized and analysed in Cytoscape to establish PPI network. And, we used Network Analyzer to perform topology analysis and confirm hub targets.

### GO function and KEGG pathwayenrichment analysis

Drug targets usually have specific biological functions, and it is important to analyze the cell component, molecular function, biological process, and biological pathways of target for predicting potential active compounds. Gene Ontology (GO) function enrichment elaborated the important functions of proteins from cell component (CC), molecular function (MF), and biological process (BP). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis show the network pathway of a particular set of genes. DAVID (Functional Annotation Bioinformatics Microarray Analysis, Version6.8,https://david.ncifcrf. gov/) provides a comprehensive set of functional annotation tools for investigators to understand biological meaning behind large list of genes[22]. Therefore, the common targets were imported into DAVID database to perform GO enrichment and KEGG pathway enrichment analysis. The species was only restricted to Homo sapiens. And the P value and the false discovery rate (FDR) were screening conditions. Finally, the main results of GO and KEGG pathway enrichment analysis were visualized as bubble plot by a free online data analysis platform— OmicShare (http://www.omicshare.com/).

### Molecular docking verification

To validate the binding ability of between the hub targets and core active compounds, AutoDock Vina software (version 4.2, http://vina.scripps.edu/) was used to perform the molecular docking program[23]. We got the structure of the hub targes from RCSB PDB (http://www.rcsb.org/)[24]. 3D structure files of core active compounds were downloaded from TCMSP or PubChem (https://pubchem.ncbi.nlm.nih.gov/) [25]. Then, we used the PyMOL Molecular Graphics System (version 2.5.0, http://www.pymol. org/) to remove the original ligands and water, and add hydrogens to the proteins[26]. The proteins and ligands were converted into “PDBQT” format, which were imput to AutoDock Vina to conduct molecular docking. The results were imported into PyMOL for visualization and analysis of the interaction and binding mode of the active compounds.

### Experimental Validation

### Main reagents and instruments

Main reagents and instrumentslisted in Supporting Information Table S1.

### Animals and Housing

48 Sprague Dawley (SD) healthy male rats (4-6 month-old, weighted 190 ± 20 g) were purchased from (license number: scxk (Zhe) 2019–0002). Then the rats freely ingested food and water and were allowed 1 week to acclimate to the standard laboratory conditions (12h light-dark cycle，23 ± 1°C and 60 ± 5% humidity).

### JDHXD Preparation

The JDHXD contains 10 herbs obtained from the Affiliated Hospital of Jiangxi University of traditional medicine and were fully validated by Medical Products Administration of Jiangxi Province. The JDHXD was prepared according to the traditional Chinese decoction method. 10 herbs were kept in the arenaceous boiler and soaked for 40 min in 8 volumes of cold water before boiling. The mixture was then boiled for 40 min and filtered. The first filter liquor was kept, and the herbal materials were resuspended in 6 volumes of cold water, boiled for further 30 min and filtered. The two filter solutions were mixed together and concentrated 0.75 g/ml for the JDHXD group. In addition，Positive control drug（Qianliekang Tablets, NMPA approval No. Z33020303, 0.57g) was ground and added to the same amount of therapist for Qianliekang-treated group.

## CP/CPPS Model Induction

SD male rats was castrated and injected estradiol benzoate (EB) (0.25 mg/ kg/ day, s.c.) for 30 days to establish EB-inducted CP/CPPS model accordance with literatures[27–29]. The detailed steps are as follows: firstly, rats were anaesthetized with 2.5% pentobarbital sodium (0.3ml/100g, i.p.), fixed in a supine position on a heating pad, and the skin of the lower abdomen and scrotum was shaved and disinfected before the surgery. Anesthetized rat was sterilized with iodophor in lower abdomen and cut with a longitudinal incision about 0.5 cm in length along medioventral line. The testicle was extruded from the scrotum on both sides, and the spermatic cord was cut after ligation. Then, sutured skin, disinfected and bandaged wound. The awakened rats were routinely fed. The wounds were wiped with iodophor every day and penicillin 200,000 U /kg was injected intramuscularly for one week. After castrating, estradiol benzoate (0.25 mg/ kg/ day, s.c.) were multipoint hypodermic injected into the back on day 2 for 30 days.

## Experimental Design and Test Protocol

After the castration surgery，all rats were adaptive feeding 7 days. Then, those rats were randomly divided into six groups with 8 rats in each group:

Normal group (Normal): the rats were given subcutaneous injection of 0.25 mg/kg distilled water on the back, continuously for 30 day, then given distilled water at a dose of 100mg/kg/d by oral gavage for 30 days；

Model group (Model): the rats were induced into CP/CPPS model and daily given distilled water at a dose of 1g/kg/d by oral gavage for 30 days;

Qianliekang table-treated group (QLKT): CP/CPPS rats were given Qianliekang table at a dose of 0.45g/kg/d by oral gavage for 30 days;

Low-dose JDHXD group (JDHXD-L):CP/CPPS rats were given JDHXD at a dose of 5g/kg/d by oral gavage for 30 days;

Middle-dose JDHXD group (JDHXD-M): CP/CPPS rats were given JDHXD at a dose of 10g/kg/d by oral gavage for 30 days;

High-dose JDHXD group (JDHXD-H): CP/CPPS rats were given JDHXD at a dose of 20g/kg/d by oral gavage for 30 days.

At the end of model establishment, a mouse was randomly selected from normal group and model group, which used to confirm the successful establishment of prostatitis model by Histological analysis. At the end of the experiment, all rats were sacrificed, and the prostate and serum samples were collected for subsequent analysis.

### Histological analysis

Rat's prostate tissues were soaked and fixed in 10% neutral formalin for 24h，then eluted with PBS solution 3 times, 4 minutes each time. The fixed tissues were dehydrated and embedded in paraffin, which were sliced into 3μm paraffin sections and stored at 4℃ for later use. After that, Hematoxylin and eosin (HE) stain was performed to observe the tissue and cytological changes such as inflammatory cell infiltration level, necrotic tissue retention and shedding, and interstitial edema in the prostate tissue under an optical microscope.

### Quantitative Real-time PCR detect MAPKs signaling pathway related mRNA (JNK, ERK1/2 and P38) expression level

According to manufacture's instructions, we used Trizol reagent to extract total RNA of 100 mg rat's prostate tissues stored at -80℃. And reverse transcription was performed to synthesize cDNA, which was then amplified target gene JNK, ERK1/2 and P38 mRNA by PCR. The information of primers sequence listed in Table 2. Then, the amplified products were subjected to 1.5% agarose gel electrophoresis. The gray value of each band was analyzed by MIAS-2000 image analysis system, and the relative content of mRNA was expressed as semi-quantitative RT-PCR results by the ratio of sample targets gene to the Internal Control (GAPDH). The $2^{-\Delta\Delta}$Ct (Livak) Method is used to perform relative gene expression analysis[30].

### Table 2. The information of primers sequence

| Gene | Sequence（5’-3’） （F:Forward;R:Reverse） |
|---|---|
| GAPDH | F:ATCAACGGGAAACCCATC |
| | R:GAAGACGCCAGTAGACTCCA |
| JNK | F:CGCCATTCTTAGTTCGCTCC |
| | R:CTCTCCAGCACCCGTACATC |
| ERK1/2 | F:TCATAGGCATCCGAGACAT |
| | R:GCTCAGGGTCAGCAATCC |
| p38 | F:TTGGACTCGGATAAGAGGATCAC |
| | R:TAGGTCAGGCTCTTCCATTCG |

### Protein expression of p-JNK, p-ERK1/2 and p-P38 detected with Western Blot

The total protein concentration in the tissue homogenates was determined using a BCA protein assay kit. The first step of the sample preparation is disruption of the sample's cellular structure by grinding and cell lysis reagents (SDS). After centrifugation, the supernatant was transferred to a new 1.5 mL centrifuge tube and stored in -80℃. In addition, a 200 ul centrifuge tube was divided into a small amount of supernatant for protein concentration determination to avoid repeated freeze-thaw. Based on the Protein assay technical handbook, we prepare a set of protein standards for determining the concentration of proteins in solution to calculate the concentration of read the value at 570 nm. Calculated the protein volume required for detection according to the protein concentration determined by BCA, which were boil at 100℃ for 5 min to reduce and denature protein. Then, the proteins were separated by electrophoresis with 10% SDS polyacrylamide. After electrophoretic, transferring the proteins from the gel to the membrane (PVDF), overnight at 4℃ using 5% blocking solution. Shake and wash 3 times in PBST (PBS, 0.05% Tween 20, pH 7.4) at room temperature, rabbit anti-rat p-JNK, p-ERK1/2, p-P38 and GAPDH (1:1000,TBS), incubate at room temperature for 2 h, HRP-linked secondary antibody (1: 2000）Incubate at room temperature for 2 h, add developing liquid to photograph. The gel analysis system imaging was used to quantitatively analyze the Western blot results.

### Statistical Analysis

The GraphPad Prism 8.0 was used for data analysis. The results were expressed in the form of mean ± standard deviation (SD). Student’s t-test, one-way ANOVA or two-way ANOVA methods were used. Statistically significant differences were defined as $p<0.05$.

# Results

### Active compounds, corresponding targets and CP/CPPS related targets

In this study, we identified a total of 143 drug ingredients with the criteria of OB and DL value. In addition， 6 main components were included (Emodin, Geniposidic acid, Vaccarin, Cardamomin, Cyasterone, and Norharman), although their OB or DL did not meet the criteria. Ultimately, we identified a total of 149 compounds as potential active compounds. And 1112 targets of those active compounds were collected from TCMSP and ETCM database. The information of 149 active compounds of 10 Chinese herbs in JDHXD were shown in Table S2. The corresponding targets of 149 active compounds were shown in Table S3.

909 potential CP/CPPS related targets were identified by GeneCards, OMIM and DisGeNET databases. By mapping compound targets to disease targets, we obtained 161 common targets, which associated with 136 compounds in JDHXD. The intersection Venn diagram is shown in Figure 2(B).

### Herbs-Components -Targets network construction and analysis

We imported information about 10 herbs，136 active compounds，and 161 common targets into Cytoscape software to construct the components - targets network (Figure 2A ). This network contained 308 nodes and 1759 edges. MOL000098 (quercetin) had the highest number of targets and could interact with 95 target proteins. From the point of view of targets, PTGS2 has the highest connectivity, targeting 114 compounds. The average number of degree is 11.52. Among them, a compound interacts with multiple targets and different compounds also act on a target at the same time. The compounds with more than 2-fold of the median degree were extracted from the network. So, we obtained 5 core active compounds.

### PPI analysis

We obtained PPI relationships for common targets in the STRING database. The result of PPI analysis was imported to Cytoscape to construct PPI network. The visualized graph is shown in Figure 2C. The PPI network was enriched to 3583 edges. Nodes with greater size and brighter color were higher degrees. According to topological analysis, the average degree of all nodes was calculated as 44.51. The top 10 connectivity degrees in the network are respectively TP53, AKT1, IL6, MAPK3, VEGFA, JUN, CASP3, TNF, and MYC. Those nodes are important nodes in the whole network and may play important biological functions in protein interaction. The nodes with more than 2-fold of the median degree were extracted from the network. So, we obtained 18 hub targets.

Setting P value <0.01 and FDR <0.01 as statistically significant. We obtained GO function 277 items (206 BP, MF48, CC 23) and KEGG pathway 114 items. The Top 20 GO functions and KEGG signaling pathways are shown Figure 3. Biological processes were principally linked to positive regulation of transcription from RNA polymerase II promoter, response to drug, signal transduction, inflammatory response, oxidation-reduction process, MAPK cascade, etc. Cell components were mainly involving nucleus, cytoplasm, cytosol, plasma membrane, etc. Molecular functions were mainly including protein serine / threonine kinase activity, protein tyrosine kinase activity, kinase activity, protein binding, ATP binding, enzyme binding, etc. JDHXD may regulate inflammatory response, immune stress, reduction process of

oxidation, MAPK Cascade and other biological processes at these cell components, and binding to cytokines and related receptors, receptor ligands and protein ligases to have therapeutic effect. KEGG signaling pathways were revealed pathways in cancer, Hepatitis B, MAPK signaling pathway, PI3K-Akt signaling pathway, TNF signaling pathway, and so on. These pathways are involved in inflammatory response, injury repair, oxidative stress, and immune response, which are closely related to the development of CP/CPPS.

### Molecular docking

We selected top 5 hub targets (TP53, AKT1, IL6, MAPK3, VEGFA) for molecular docking with 5 core active compounds (quercetin, luteolin, emodin, formononetin, kaempferol). The predicted value of AutoDock Vina indicates the binding activity: the binding affinity values <0 kcal/mol indicate that the ligand can bind to the receptor; binding affinity values ≤ −5.0 kcal/mol show a better binding ability. The binding energy of top 5 hub targets and 5 core active compounds showed in table 3. In this study, the top 5 hub targets were well binding with 5 core active compounds. The lower affinity value was, the higher the affinity between targets and compounds, indicates that these principal components may act on hub proteins to play a certain regulatory role. Figure 4 showed schematic 3D representation that the molecular docking model, active sites, and binding distances between 5 core active compounds and 5 hub targets.

**Table 3. The binding energy of top 5 hub targets and 5 core active compounds (kcal/mol)**

| Target | Quercetin | Luteolin | Formononetin | Kaempferol | Emodin |
|---|---|---|---|---|---|
| IL-6 | -6.7 | -6.7 | -6.7 | -6.7 | -6.4 |
| TP53 | -6.8 | -6.7 | -6.1 | -6.3 | -6.1 |
| AKT1 | -7.9 | -8.2 | -8.0 | -7.8 | -8.3 |
| MAPK3 | -7.8 | -7.8 | -7.1 | -8.3 | -8.3 |
| VEGFA | -6.0 | -5.8 | -5.8 | -6.0 | -6.1 |

### Effect of JDHXD on histopathological features in CP/CPPS rat model

As shown in Figure 5, histological analysis revealed that the epithelial cells were cuboid, the exudation of glandular lumen were few, and tissue edema and inflammatory cells were not found in the lumina of the prostate from rats in the normal group. Compared with normal group, there some histopathological features in the prostate of CP/CPPS model: the mesenchyme tissues became sparse and edema; the inflammation consists predominantly of infiltrations of plasma cells and lymphocytes into the glandular lumina and stroma were readily apparent; dilatation and hyperaemia of vascular and a small amount of mast cells fibroblasts were observed; the papillary hyperplasia of acinar epithelial cells was obvious. These pathological features of inflammation confirmed that the model was successfully established,

which is consistent with the description in literatures. Whereas, both the positive drug and JDHXD treatment alleviated the EB-induced inflammation in rat prostate. Significantly decreased infiltrating plasma cells and lymphocytes were observed in JDHXD-treated groups than the positive drug group. (HE, × 100)

### Effects of JDHXD on the activities of MAPK signaling pathway

We detected key gene expression in three major pathways in the MAPK signaling pathway by qRT-PCR and Western blot. As shown in Figure 6, the tissue mRNA expression level of JNK, ERK1/2 and P38 increased significantly in model group compared with normal group ($P < 0.05$). Both the positive drug and JDHXD significantly decreased these genes expression compared to the model group ($P < 0.05$). Treatment with JDHXD at 5 g/kg, 10 g/kg, and 20 g/kg decreased these specific inflammatory cytokine levels in an approximate dose-dependent manner. In Figure 7, western blot experiment showed that the expression levels of p-JNK, p-ERK1/2 and p-P38 was significantly higher than the normal group ($P < 0.05$), which mean MAPK signaling pathway was activated in the EB-induced CP/CPPS rat model. After treatments, expression levels of p-JNK, p-ERK1/2 and p-P38 significantly lower than the model group ($P < 0.05$), which indicated that QLKT and JDHXD had protective effect on CP. Further analyses revealed that high-dose JDHXD group significantly down-regulated the phosphorylation of MAPK signaling pathway than positive drug control group (QLKT), which suggested that JDHXD has a better protective effect on CP/CPPS. All of the raw data using western blotting were supplemented in Figure S1.

## Discussion

Currently, there are no standard treatments to alleviate symptoms for CP/CPPS. Weak evidence supports the use of the therapeutic interventions (such as antibiotics, anti-infammatory medications, pain medication and alpha-blockers) for CP/CPPS. Traditional Chinese drugs have been applicated to treat urologicaldiseases with more than 1,000 years. Multiple components in the herbal formulae often play a synergistic role that is greater than individual drug. Because of herbal formulae's feature of multi-compounds multi-targets in the treatment of complex diseases, traditional Chinese drugs therapeutics have been paid increasing attention in recent years. And someclinical studies provided evidence manifested thatherbal formulae could be equipped with the safety, efficacy and high compliance for CP/CPPS[31]. Examination of more than 5,000 evidence-based clinical studies provides substantial evidence that Chinese medicine can effectively treat chronic prostatitis[32,33]. JDHXD was developed based on decades of clinical experience, clinical and experimental studies have proved its effectiveness for CP/CPPS. In the theories of Chinese medicine, it has he functions of clearing heat, disinhibiting dampness, resolving toxin, activating Qiand blood circulation and relieving pain. Our group previously found that JDHXD can reduce the levels of proinflammatory cytokines IL-6 IL-8 in prostatic fluid (EPS) of patients with CP/CPPS ,and protect against chronic prostatitis in rat model through NF-κB signaling pathway[8].

In this study, we collected various active compounds (quercetin, luteolin, emodin, formononetin, kaempferol, etc.) based on databases. These active compounds mainly contain flavonoids, polyphenols, polysaccharides, organic acids and so on. Some of potential active compounds have been shown to have a therapeutic effect on CP/CPPS respectively. Such as, quercetin，a kind of bioflavonoids，can reduce the expression of pro-infammatory cytokines, improve antioxidant ability, and more importantly suppress the phosphorylation of NF-κB and MAPKs in CP/CPPS rat model[34]. Shoskes et al. found the bioflavonoid quercetin was well tolerated and provides significant symptomatic improvement in most men with CP/CPPS in a prospective randomized, double-blind, placebo-controlled trial[35,36]. Moreover, quercetin can alleviates neuropathic pain by mediating AMPK/MAPK pathway in rats with chronic constriction injury[37].

Latest research has shown that kaempferol exhibit its anti-oxidant and anti-inflammatory effects through inhibiting the phosphorylation of ERK-1/2, p38, JNK, and activation of NF-κB induced by IL-1β[38,39]. kaempferol can inhibit the overproduction of proinflammatory cytokines, including TNF-α, IL-1β and IL-6, and can strongly reduce the activation of MAPKs and NF-κB signaling pathways in LPS-induced acute lung injury in mice[40]. In addition, Kaempferol, with its androgenic-like activity, served as a selective androgen receptor modulator that contributes to androgen-related BPH development[41]. Likewise, emodin could inhibit the activation of NF-κB, JNK, p38MAPK , suppressing the expression of TNF-α, IL-6, IL-1β, and MMP-9 to anti-inflammatory activity in the treatment and prevention of various inflammatory disorders[42,43]. In vitro, vivo, silico, and clinical studies strongly suggest that the major pharmacological mechanism of luteolin is its anti-inflammatory activity, which derives from its regulation of inflammatory mediators IL-1β, IL-2, IL-6, IL-8, IL-12, IL-17, TNF-α, interferon (IFN)-β, and granulocyte-macrophage colony-stimulating factor and increase the level of IL-10[44–46].

We predicted several targets (TP53, AKT1, IL6, MAPK3, VEGFA, JUN, CASP3, TNF, etc.) of JDHXD for CP treatment based on network pharmacology. Many of these targets have been shown to be closely associated with CP/CPPS, such as IL-6, AKT1, TNF. Overexpression of inflammatory cytokines and chemokines such as IL-1β, TNF-α, IL-2, IL-6, IL-8, MCP-1, et al have beenobserved in clinical samples from CP/CPPS patients[47–52]. These indicators released after inflammatory injury, which may increase the sensitivity of peripheral afferent nerves to stimulation and cause chronic pain[53–55]. IL-1β, IL-6, IL-8, and TNF-α positively correlated with the Self Anxiety Scale (SAS) and the Self Depression Scale (SDS). IL-10 and TNF-α were significantly and positively correlated with NIH Chronic Prostatitis Symptom Score (NIH-CPSI) in patients with CP/CPPS[56]. In EPA (Experimental autoimmune prostatitis)-induced rat model, the expression of IL-1β, IL-6, and TNF-α in prostate tissues or serum significant increased. Overexpressed the inflammatory factors, such as IL-1 β, IL-6 and TNF-α play an important role in the activation of immune system and inflammatory infiltration of EAP rats[57–60]. In addition, the study shown patients with CP/CPPS are more likely to have a low IL-10 producing genotype, suggesting low TNF-α and high IL-10 phenotypes may lead to anti-inflammatory phytotherapy failure[61]. Animal model and clinical studies indicated that androgen receptor insensitivity or dysfunction might play certain role in the pathogenesis of CP/CPPS[4]. VEGFA were found to be abnormally high expression in prostatitis rat models[62]. In our previous experiment, the tissue inflammatory factor levels of IL-1β, IL-2, IL-6, and TNFα increased

significantly in EB-induced CP/CPPS rats[8]. JDHXD could reduce or eliminate the inflammatory response of the rat prostate, improve the pathological damage of the tissue structure, and play a role in the repair and protection of the prostate tissue through down-regulating inflammatory factor[7].

KEGG pathway enrichment analysis reveal JDHXD may exerts its effects by regulating those signaling pathways, including the MAPK signaling pathway, PI3K-Akt signaling pathway, TNF signaling pathway, and so on. In various common rat models of CP/CPPS, many signaling pathways were found overactivation, such as PI3K/Akt/mTOR pathway, NF-κB pathway, MAPKs signaling pathway[34,56,63,64]. Intervention of these important biological pathways is an important pharmacological pathway for some therapeutic drugs. PI3K/AKT signaling pathway was closely related to the development of inflammation and oxidative stress in CP/CPPS[63]. NF-κB is one of the most important transcription factors and plays a role in the expression of pro-inflammatory genes, including cytokines, chemokines, and adhesion molecules[65]. NF-κB was activated in EB-stimulated prostate revealed in our previous studies. And JDHXD regulated the expression of NF-κB and its inhibitor Iκ-Bα[8]. MAPK signaling pathway is an important transmitter of extracellular signals from the cell surface to the nucleus, which could be activated by oxidative stress and a variety of pro-inflammatory factors. Experiments have shown that MAPK signaling pathway was activated by increased phosphorylation of ERK1/2、JNK and P38 to mediate the production of a variety of pro-inflammatory factors[34]. In our experiment, the expression levels of JNK, ERK1/2 and P38 was significantly increased and JDHXD with different concentration gradient could obviously inhibit the expression of JNK, ERK1/2 and P38, which would further regulate the expression of various pro-inflammatory cytokines.

Taken together, our results suggest that JDHXD contains multiple components, which can act on multiple targets and regulate cytokines in different signaling pathways through different biological processes to play a therapeutic role. But the present study has several limitations. Only relying on the target acquisition, network calculation and biological enrichment results of the database platform cannot accurately and reliably reveal the drug-regulated disease network. The network pharmacological prediction results must be verified by both pharmacological experiments and clinical studies, which obtained the final screening results would be real and useful.

# Conclusion

This study demonstrated potential mechanism of JDHXD in treating CP/CPPS. Network pharmacological revealed the material basis and mechanism of JDHXD in the treatment of CP/CPP are that JDHXD contains numerous active compounds (flavonoids, polyphenols, polysaccharides, etc.), which synergistically regulate multitarget (TP53, AKT1, IL6, MAPK3, VEGFA, etc.). JDHXD effectively treats CP/CPPS by regulating the key pathways of inflammatory, including MAPK, NF-κB, PI3K-Akt signaling pathways, etc. Combined with previous studies and this experimental verification, we hold the opinion that JDHXD could inhibit or activate some target proteins of MAPK and NF-κB signaling pathways to downregulate of serum IL-6, IL-1β, TNF-α, and other cytokines levels. In terms of study design and

methodology, the reliability and effectiveness of those methods are very helpful to explore the material basis and mechanism of traditional Chinese medicine.

# Abbreviations

JDHXD: Jiedu Huoxue decoction

CP/CPPSu: chronic prostatitis/chronic pelvic pain syndrome

GO: Gene Ontology

KEGG: Kyoto Encyclopedia of Genes and Genomes

PPI: Protein-protein interaction

EB: estradiol benzoate

OB: oral bioavailability

DL: drug likeness

# Declarations

### Ethics Approval and consent to Participate

The animal protocol was approved by the Animal Use and Care Committee of the Jiangxi University of Chinese Medicine (Nanchang, China, Permission No. 323-07-01339/2019-09/01). We confirm that experiments were performed in accordance with relevant guidelines and regulations. And all methods are reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

### Consent for Publication

Not applicable

### Availability of data and materials

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

### Competing interests

The authors report no conflicts of interest in this study.

### (e) Funding

This study is supported by Jiangxi university of traditional medicine 1050 youth talent project (Grant Number: 5142001001)Jiangxi Science, Technology and Innovation Commission (No. 201904010234); National Natural Science Foundation of China (No. 81903963)

### (f) Acknowledgment

Not applicable.

### (g) Author's Contribution

Yan Zhangren designed the study.

Pan Junqing and Zhang Sina performed the network pharmacology analyses and molecular docking analysis.

Hu XiangJun, Deng Siqiao, Zhang Congzhong conducted the experiments;

Yin Hongwei and Liu Can performed the statistical analyses;

Pan Junqing wrote the manuscript and critically revised the article for essential intellectual content.

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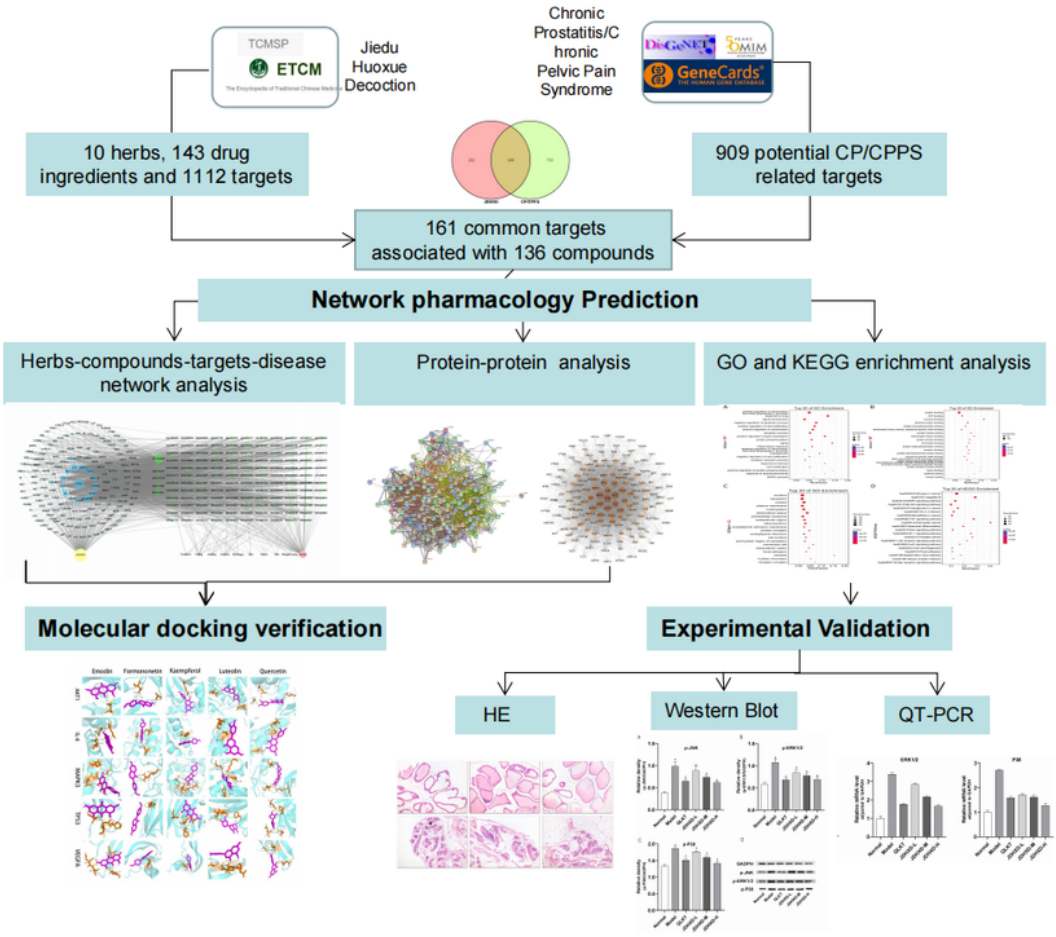
# Figures




## Figure 1

Flow chart. The whole research was divided into three steps. The first step was to construct and analysis the herbs-compounds-targets-disease network by network pharmacology. In the second step, molecular docking was used to validate the binding ability of between the hub targets and core active compounds. The third step was the validation of the key signal pathway based on animal experiment.

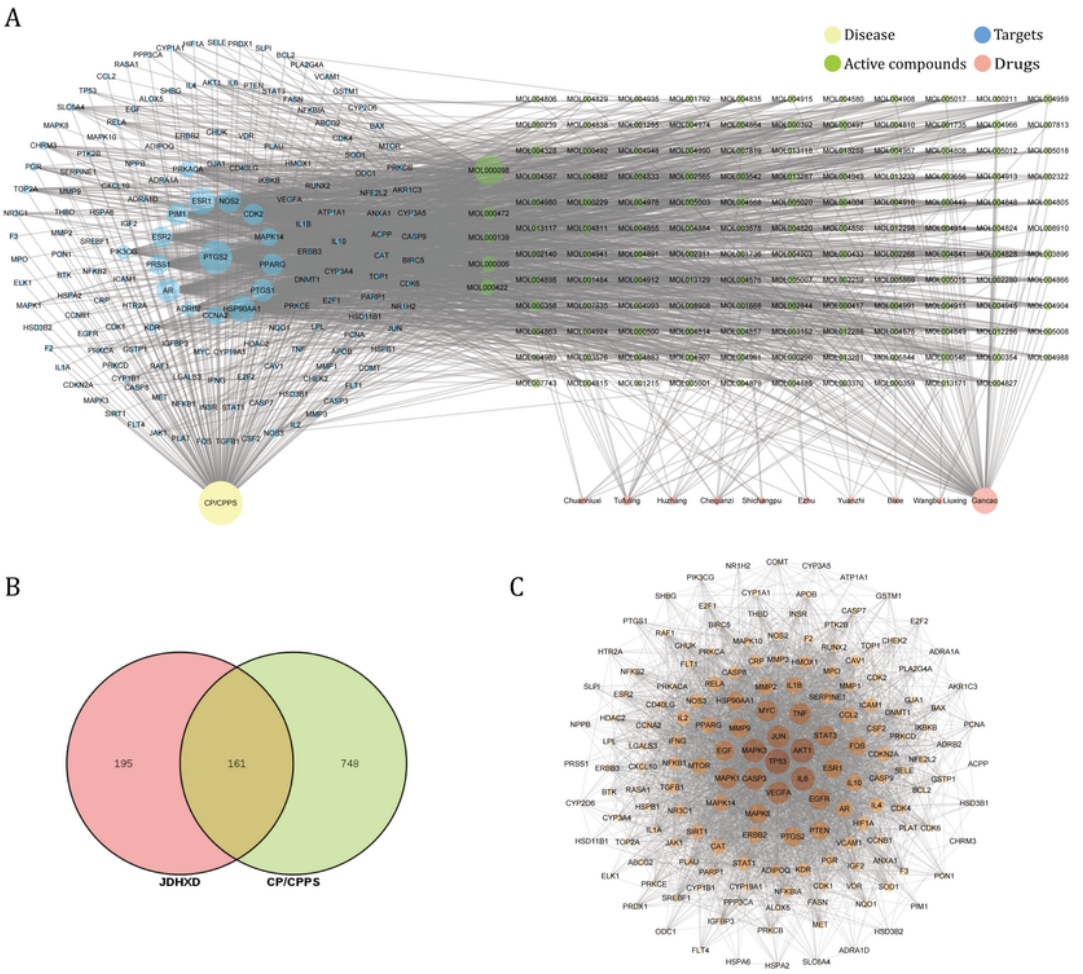
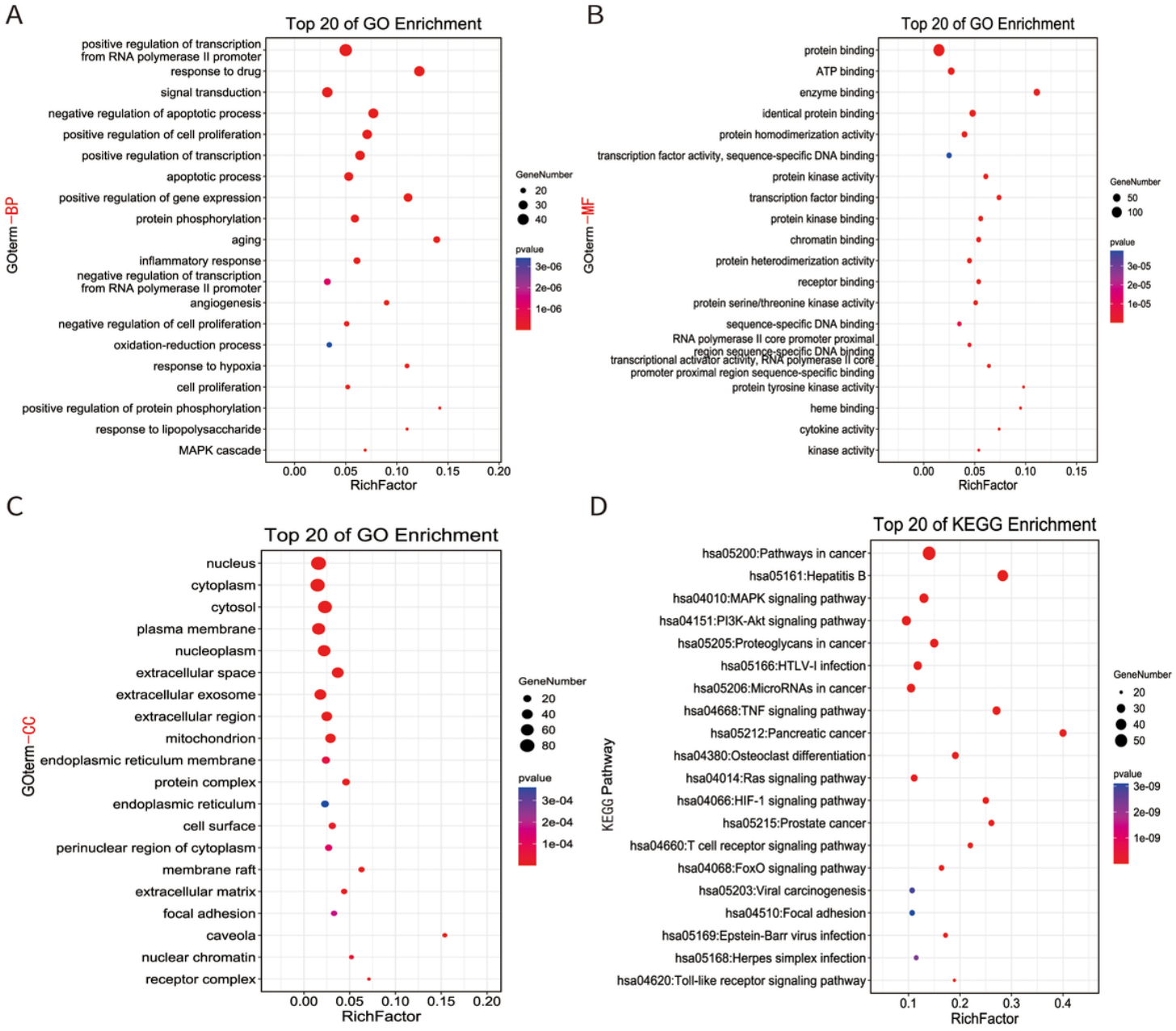



Figure 2

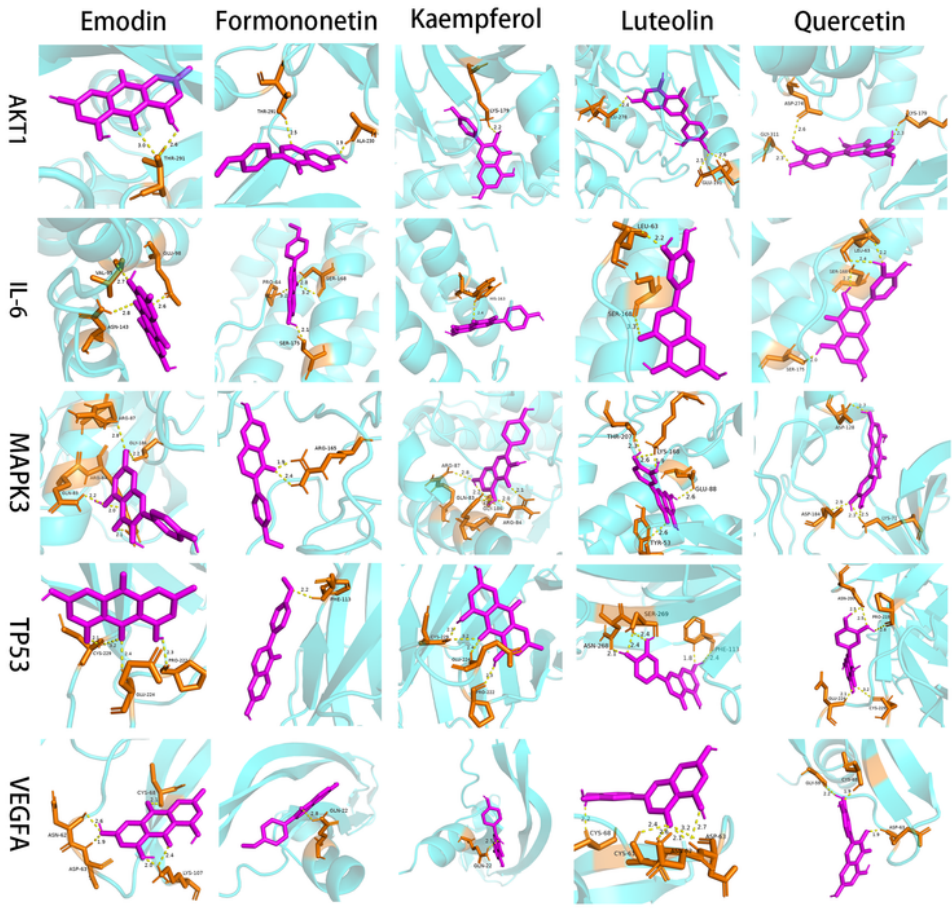
(A) Herbs-Components -Targets network. (B) Venn diagram of JDHXD and CP/CPPS related targets. The overlapping genes were selected for further analysis. (C) Protein-protein interaction Network. The size of the shape reflected the importance of protein in network analysis.

GO function and KEGG pathway enrichment analysis




## Figure 3

The top 20 of GO function and KEGG pathway enrichment. (A) BP enrichment analysis. (B) MF enrichment analysis. (C) CC enrichment analysis. (D) KEGG enrichment analysis. The size of each node indicates enriched numbers, the abscissa represents the enriched RichFactor, and color means enriched adjusted P-value.




## Figure 4

**Schematic 3D representation that the molecular docking model, active sites, and binding distances between 5 core active compounds and 5 hub targets.**

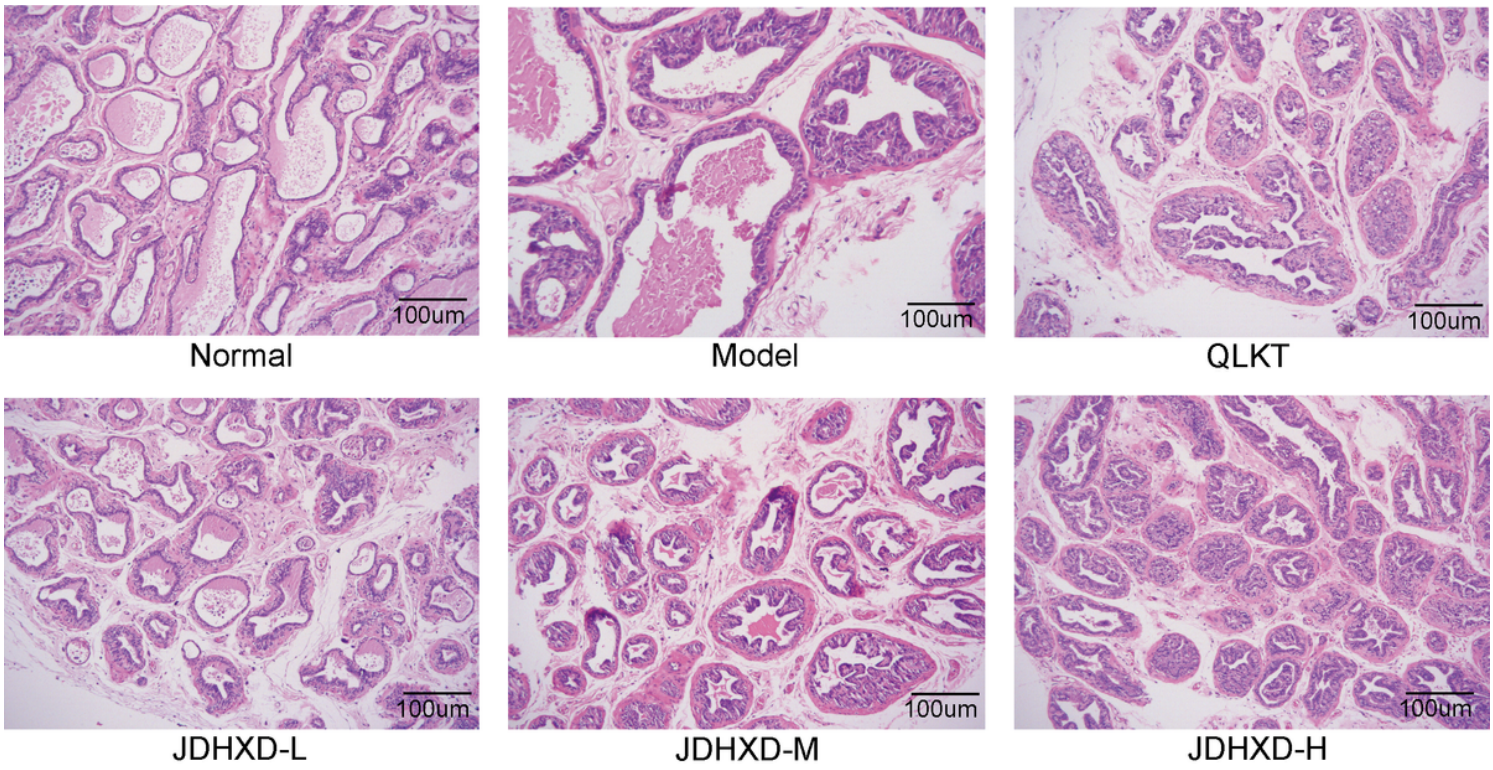



Figure 5

HE histopathological staining in each group of rats (×100)

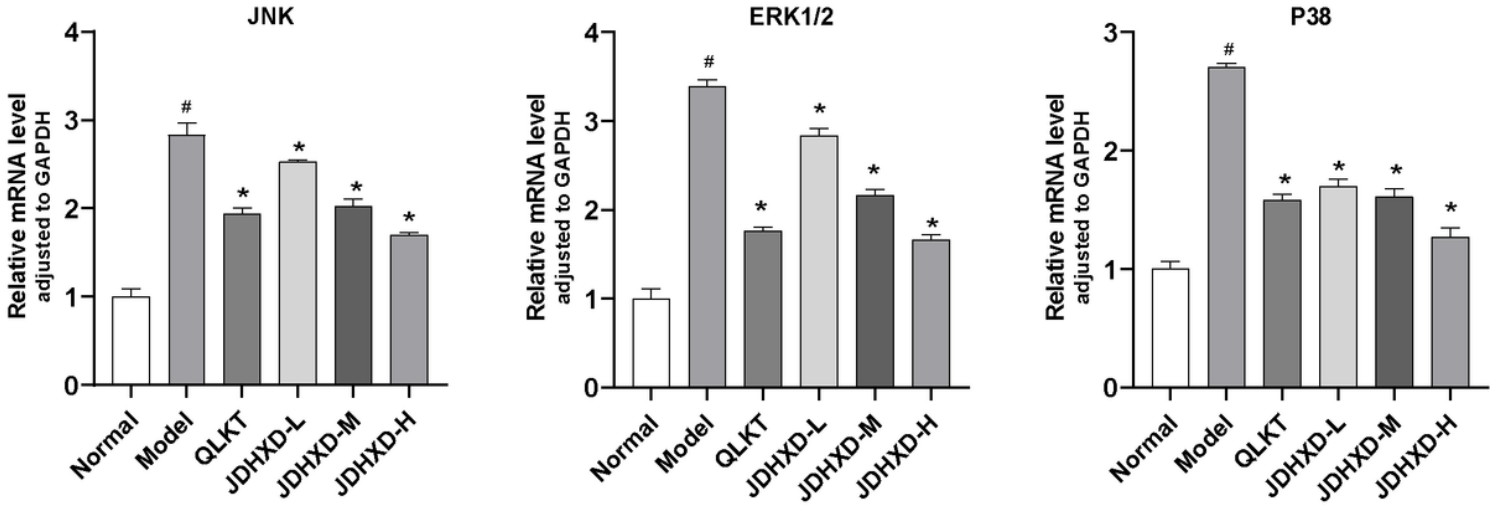



Figure 6

Histogram of JNK, ERK1/2, P38 mRNA expression detected with qRT-PCR. *p < 0.05 compared with model group, #p < 0.05 compared with normal group.

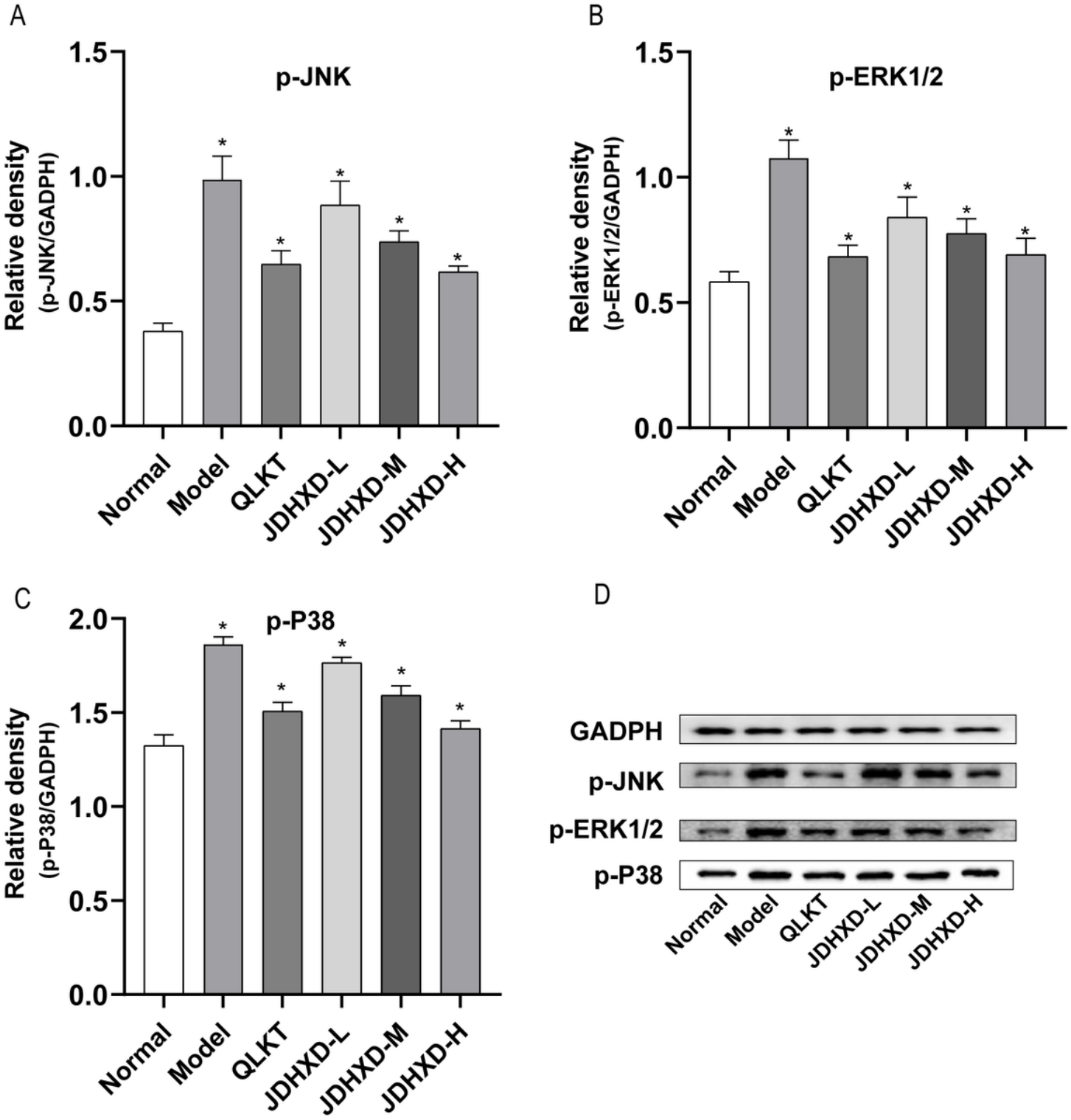



Figure 7

(A-C) The relative density of GADPH, p-JNK, p-ERK1/2, and p-P38 proteins were determined by Western blot analysis. *p < 0.05 compared with model group, #p < 0.05 compared with normal group. (D)The expression of gray values were calculated.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.Mainreagentsandinstruments.docx
- TableS2.Theinformationof149activecompoundsof10ChineseherbsinJDHXD.xls
- TableS3.Thecorrespondingtargetsof149activecompounds.xlsx
- FigureS1.Alloftherawdatausingwesternblotting.pdf