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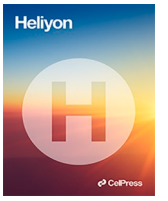

Research article

Elucidating Cyathula Officinals' mechanism in osteoarthritis treatment: Network pharmacology and empirical evidence on anti-inflammatory actions

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ABSTRACT

In this study, we explored the therapeutic potential of Cyathula Officinals (CNX) in Knee Osteoarthritis (KOA) treatment. Utilizing network pharmacology and in vitro experiments, we identified active ingredients, action targets and pathways in CNX. Our analysis, integrating databases like TCMSP, SwissTarget Prediction, Genecards, CTD, STRING, and DAVID, highlighted 396 action targets and 283 disease targets, pinpointing 64 intersection genes linked to KOA. The significant involvement of the MAPK and NF-κB pathways in CNX's anti-inflammatory action was validated through qPCR, which might underlie CNX's efficacy in inhibiting chondrocyte apoptosis and IL-6 expression. These findings suggest CNX's potential in KOA management, offering insights for its clinical application.

1. Introduction

Knee Osteoarthritis (KOA) is a common chronic articular disease intimately intertwined with the process of aging, physical overexertion, obesity, and congenital joint anomalies. This debilitating ailment precipitates the progressive degeneration of the articular cartilage, subsequently instigating a spectrum of clinical manifestations encompassing pain, swelling, deformity, and dysfunction of the knee joint [1]. KOA is a widespread affliction, notably affecting the demographic strata of middle-aged and elderly individuals, with its global prevalence demonstrating a year-over-year growth [2]. As society enters an era characterized by the high prevalence of various diseases [3–7] among the vulnerable population, the worldwide populace grappling with osteoarthritis exceeds a staggering 300 million [8].

Knee osteoarthritis could induce structural metamorphoses in the knee joint, heralding the gradual degradation of not only the cartilage but also the menisci and ligaments, coupled with inflammation of the synovial membrane [9]. The wear and degeneration of cartilage emerge as the primary instigators of the inflammation. The cartilage comprises chondrocytes and an extracellular matrix (ECM), wherein the type II collagen, fibrous proteins, proteoglycans, hyaluronic acid, and chondroitin sulfate represent the principal constituents [10]. The pro-inflammatory cytokine IL-1βhas been elucidated to incite the synthesis of inflammatory mediators and orchestrate the degradation of type II collagen, ultimately culminating in the apoptosis of chondrocytes. The contemporary medical

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approaches encompass the utilization of non-steroidal anti-inflammatory drugs, corticosteroid injections, and tramadol to alleviate symptoms, but their prolonged administration is often constrained due to the side effects [11].

Cyathula Officinals (Chuaniuxi, CNX) is a traditional medicinal plant that is frequently employed for the treatment of osteoarthritis, rheumatic, gynecological, and blood stasis disorders [12]. Recent investigations have unveiled the anti-inflammatory and immunomodulatory attributes inherent to CNX([13,14]). Particularly, Park et al. have demonstrated the ability of CNX extract to attenuate MMP-13 expression in IL-1β-stimulated SW1353 cells, implicating its potential role in the inhibition of chondrocyte apoptosis [15].

Another research finding alluded to CNX's rich reservoir of active constituents, which manifest anti-inflammatory and analgesic properties [16]. Additionally, further investigation revealed that CNX's therapeutic mechanism of action in treating KOA involves the modulation of several critical signaling pathways, such as the NF-κB and MAPK pathways, which are recognized for their involvement in inflammation and pain [17,18]. Zhao et al. elucidated the inhibitory effects of ethanol extract of CNX on the activation of the MAPK signaling pathway [19]. Similarly, Yang et al. unveiled the ability of a range of cyasterone derivatives derived from CNX to regulate the

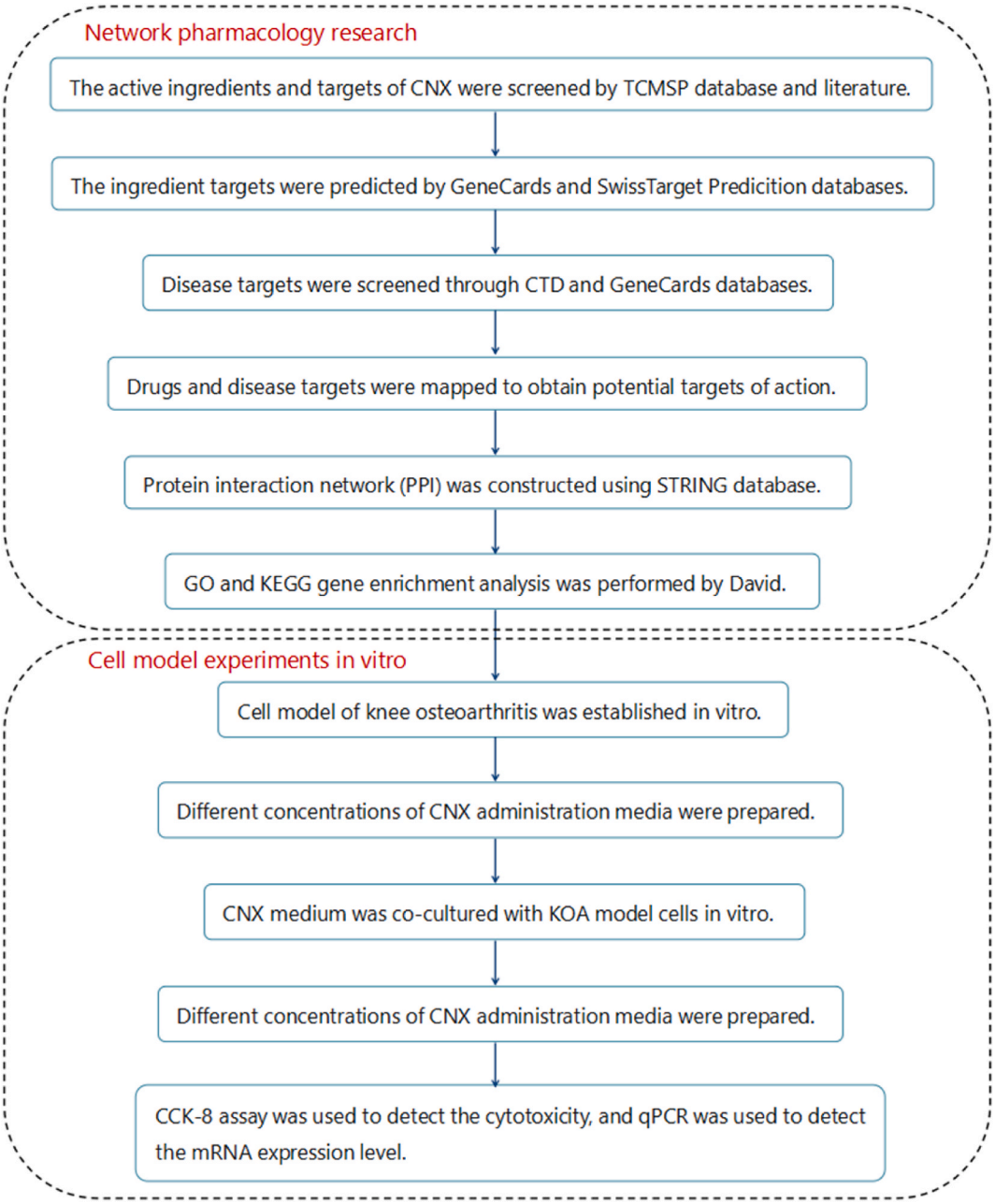

Fig. 1. Graphical Abstract The pharmacological mechanism underlying CNX's efficacy against KOA is elucidated through a combination of network pharmacology and experimental validation techniques.

NF-κB pathway, concurrently inhibiting the release of nitric oxide (NO) from LPS-stimulated RAW 264.7 cells [20].

Despite numerous studies examining the individual components of CNX extracts, the comprehensive therapeutic effect and mechanism of CNX remain unclear. The objective of this study is to employ network pharmacological methods to analyze the active ingredients, action targets and signaling pathways of CNX in order to investigate its potential correlation with knee osteoarthritis (KOA).

The network pharmacological approach is a technique that integrates biological and pharmacological data to identify the active ingredients and action targets of drugs, as well as to predict their mechanisms of action (as illustrated in Fig. 1). Through an analysis of the interactions between the active ingredients, targets, and signaling pathways of CNX, this approach aims to comprehend its potential efficacy in treating KOA. Through an examination of the interconnectivity among the active constituents, targets, and signaling pathways of CNX, this investigation endeavors to furnish a theoretical framework for future inquiries into the anti-inflammatory mechanism of CNX, as well as for the advancement of novel pharmaceuticals and clinical investigations.

Utilizing network pharmacology and in vitro experiments (as illustrated in Fig. 1)., we aimed to integrate databases like TCMSP, SwissTarget Prediction, Genecards, CTD, STRING, and DAVID, highlighted the intersection of action targets and disease targets, pinpointing the intersection genes linked to KOA.

2. Materials and methods

2.1. Collection of potential targets of CNX

The Traditional Chinese Medicine Systematic Pharmacology Database and Analysis Platform (TCMSP: https://tcmsp-e.com/) was utilized to identify compounds in CNX that possess favorable attributes such as drug similarity (DL) and oral bioavailability (OB). This database is recognized as one of the most extensive collections of traditional Chinese medicine compounds. The criteria for screening active compounds were set at OB $\geq$ 30% and DL $\geq$ 0.18, which are considered to be the most crucial indicators. The keyword "Cyathula Officinals" was utilized in TCMSP to conduct a search and screening of essential active compounds. After the preliminary screening, a comprehensive analysis of the compounds was carried out either individually or by conducting literature searches. This analysis aimed to generate the final list of active compounds. The SDF format files of CNX compounds were obtained from PubChem by entering the chemical components of CNX into the database. This allowed us to retrieve the chemical structures and download the SDF format files containing the two-dimensional structures. Next, we used the SwissTarget Prediction platform (http://www.swisstargetprediction.ch/) to predict the targets of the compounds based on their two-dimensional structures. In cases where the compounds were not available on the SwissTarget Prediction website, we conducted a thorough search on TCMSP and GeneCards.

2.2. Collection of potential targets against KOA

KOA is primarily characterized by knee pain, swelling, deformity, and dysfunction. To identify relevant targets, two databases, GeneCards (https://www.genecards.org) and CTD (https://www.ctdbase.org), were utilized to search for targets using keywords such as 'Knee osteoarthritis' and 'Knee synovitis'. After merging the results, duplicate data were eliminated to obtain the disease target group. Finally, Venn diagrams were employed to analyze the intersection network of compound-target and disease-target. The approach described in the article can be valuable in identifying potential targets for treating other diseases as well.

2.3. Construction of a network of protein-protein interactions (PPI)

The identification of potential targets for the treatment of KOA involved constructing a potential target PPI network using the STRING database, which predicts protein interactions. The topological significance of nodes was evaluated based on three features: 'betweenness,' 'closeness,' and 'degree.' Essential targets were then selected based on these features. Genes that met the criteria were considered core genes, and the network was visualized using Cytoscape 3.7.2 software. This method helps in understanding the interactions between proteins and can be useful in identifying potential targets for drug development. The approach described in the article can be used to identify potential targets for other diseases as well.

2.4. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis

The categorization of gene functions and their products, encompassing biological processes, cellular components, and molecular functions, is frequently achieved through the utilization of GO analysis. The biological process category serves to define the cellular or physiological functions of the genes under investigation. The KEGG pathway analysis is a valuable tool for identifying important biological pathways by connecting genomic information with practical knowledge. In this study, we conducted the analysis by inputting the core targets obtained in Section 2.3 into DAVID 6.8. Statistical significance was determined using a p-value threshold of less than 0.05. The data was visualized using R for the bubble chart and Cytoscape 3.7.2 software for the 'Ingredient-target-pathway' network.

2.5. Experimental validation

2.5.1. Cell culture

In this investigation, primary replacement culture method was employed to isolate knee chondrocytes (chondrocytes) of SPF SD mice aged 1 week from Guangzhou Xinyuan Biotechnology. These chondrocytes were maintained in a primary chondrocyte culture system containing 10% FBS at 37 °C in 5% CO2 humidified air. The study subjects were degenerative articular chondrocytes induced by IL-1β, and the effects of CNX on the p38MAPK/NF-κB pathway of degenerative chondrocytes were examined. The culture medium of the cells was altered every other day, and upon reaching the logarithmic growth phase, the cells were subcultured. The present investigation employed the primary replacement culture method from Guangzhou Xinyuan Biotechnology to isolate knee chondrocytes from SPF SD mice aged 1 week, which were subsequently sustained in a primary chondrocyte culture system comprising 10% FBS at 37 °C in 5% CO2 humidified air. In order to examine the impact of CNX on the p38MAPK/NF-κB pathway of degenerative chondrocytes, the investigators utilized IL-1β-induced degenerative articular chondrocytes as the experimental model. The cells were maintained through regular medium changes every other day and subculturing at the logarithmic growth phase.

2.5.2. Drug preparation

A quantity of 20 g CNX was procured from Lingnan Pharmacy and subsequently immersed in 200 mL of single distilled water for a duration of 30 min. The mixture was then subjected to boiling for a period of 60 min, and the resultant extract was collected for a subsequent round of extraction. An additional 100 mL of single distilled water was introduced and boiled for a duration of 30 min. The two extracts were amalgamated and subjected to a process of concentration. The filtrate was obtained by filtering the residual liquid in the filter slag and combining the filtrate with two times. The filter screen was utilized to filter the filtrate, which was then subjected to extraction for a duration of 1 h using a water bath. The extract obtained was subjected to drying in a drying box, followed by pulverization into powder using a pulverizer, and subsequently stored in the same drying box. The resultant concentrated powder weighed 9.27 g, with a yield of 46.35%, and was refrigerated at 4 °C for future use. Prior to the experiment, the CNX was prepared at final drug concentrations of 0.01, 0.1, 1, 10, and 100 μg/mL using DMSO.

2.5.3. CCK-8 and drug administration

In order to assess the feasibility of chondrocytes subsequent to exposure to CNX and IL-1β, a cell counting Kit-8 (CCK-8) assay was executed. Specifically, chondrocytes derived from the third generation of articular chondrocytes that were cultured under normal conditions were subjected to an enzyme digestion technique and subsequently placed into 96-well plates at a concentration of $6 \times 10\hat{}3$ cells per well in a total volume of 100 μl of complete medium. Following a 24-h incubation period, the cells were subjected to IL-1β treatment at a final concentration of 10 μg/mL for an additional 24 h. Subsequently, a separate set of plates were treated with CNX at final concentrations of 0.01, 0.1, 1, 10, and 100 μg/mL, using the same treatment concentrations and methods as previously described.

2.5.4. Quantitative real-time PCR (qPCR)

Cells were seeded into six-well plates at a density of 1×10^6 cells in 2 mL medium to facilitate mRNA extraction. In each experiment, the cells were stimulated with IL-1β (10 μg/mL) with or without CNX (0.1, 1, and 10 μg/mL) for a duration of 24 h. Total RNA was extracted from chondrocyte cells using a RNA extraction kit (Tian Gen, China), and the concentration of RNA was measured before reverse transcription. The synthesis of cDNA was performed by reverse transcribing 2000 ng of total RNA using HiScript II Q RT SuperMix (Vazyme, China). The endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used, and quantification was done using SYBR (ChamQ SYBR qPCR Master Mix, Vazyme, China). The relative expression was determined using the comparison threshold cycle ($2-\Delta\Delta CT$) equation. The details of the primers used are provided in Table 1.

2.6. Statistical analysis

The experimental data underwent statistical analysis through the utilization of GraphPad Prism 9. The comparison of data from multiple groups possessing a normal distribution was executed via one-way analysis of variance. A p-value of less than 0.05 was considered as statistically significant.

Table 1
Primers used in qPCR analyses of mRNA expression.

Gene	Foward	Reverse
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
IL-6	AGTCCTTCCTACCCCAATTTCC	TGGTCTTGGTCCTTAGCCAC
MAPK1	GGTTGTTCCCAAATGCTGACT	CAACTTCAATCCTCTTGTGAGGG
MAPK8	AGCAGAAGCAAACGTGACAAC	GCTGCACACACTATTCCTTGAG
MAPK14	TGACCCTTATGACCAGTCCTTT	GTCAGGCTCTTCCACTCATCTAT
NFKB1	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG
IKBKB	CTGAAGATCGCCTGTAGCAAA	TCCATCTGTAACCAGCTCCAG
Rela	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC

3. Results

3.1. Active constituents and relevant targets of CNX

A set of 396 ingredient targets was obtained from the Traditional Chinese Medicine Systems Pharmacology database (TCMSP). We used SwissTarget Prediction screening the effective components of CNX and after the removal of duplicate values, we found 23 chemical ingredients including quercetin and achyranthes bidentata D as the main ingredients. Refer to Table .2 for details of relevant ingredient targets.

3.2. Search for KOA targets

From GeneCards and CTDs, "Knee osteoarthritis" and "Knee synovitis" were used as keywords to search for disease targets respectively. Two Venn diagrams were used to visualize the results and their relationship. After eliminating duplicate values, we obtained a total of 9200 targets related to KOA (Fig. 2), with 347 intersection targets.

3.3. PPI network construction and potential target selection

Through the process of intersecting drug targets and disease targets (as depicted in Fig. 3), a total of 64 intersection genes were identified for CNX against KOA. Subsequently, the correlation between these genes was analyzed using STRING, and a PPI visualization network was constructed (as shown in Fig. 4). Further analysis utilizing Cytoscape led to the identification of 64 potential targets that are deemed critical for the anti-inflammatory effects of CNX on KOA and knee synovitis.

Table 2
Main chemical composition of CNX.

Formular	Compound	Named	OB	DL
$C_{17}H_{12}O_6$	Betavulgarin	CNX-1	68.75	0.39
$C_{19}H_{26}O_5$	Rubrosterone	CNX-2	32.69	0.47
$C_{29}H_{50}O$	beta-sitosterol	CNX-3	36.91	0.75
$C_{15}H_{10}O_7$	quercetin	CNX-4	46.43	0.28
$C_{29}H_{48}O$	Chondrillasterol	CNX-5	42.98	0.76
$C_{53}H_{82}O_{25}$	Achyranthoside D	CNX-6	66.62	0.18
$C_{47}H_{72}O_{20}$	(2S,3S,4S,5R,6R)-6-[[(3S,4aR,6aR,6bS,8aS,12aS,14aR,14bR)-4,4,6a,6b,11,11,14b-heptamethyl-8a-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxycarbonyl-1,2,3,4a,5,6,7,8,9,10,12,12a,14,14a-tetradecahydropicen-3-yl]oxy]-4-[2-carboxy-1-(carboxymethoxy)-2-hydroxyethoxy]-3,5-dihydroxyoxane-2-carboxylic acid	CNX-7	66.62	0.18
$C_{39}H_{56}O_{12}$	Spinoside A	CNX-8	41.75	0.4
$C_{27}H_{44}O_7$	(2S,3R,5R,9R,10S,13S,14S,17S)-2,3,14-trihydroxy-10,13-dimethyl-17-[(2R,3R)-2,3,6-trihydroxy-6-methylheptan-2-yl]-2,3,4,5,9,11,12,15,16,17-decahydro-1H-cyclopenta[*a*]phenanthren-6-one	CNX-9	44.23	0.82
$C_{20}H_{18}NO_4^+$	Berberine	CNX-10	36.86	0.78
$C_{20}H_{19}NO_8S$	Berberine sulfate	CNX-11	36.86	0.78
$C_{19}H_{14}NO_4^+$	Coptisine	CNX-12	30.67	0.86
$C_{16}H_{12}O_5$	Wogonin	CNX-13	30.68	0.23
$C_{29}H_{50}O$	(3S,5R,9R,10S,13R,14R,17R)-17-[(2R,5S)-5-ethyl-6-methylheptan-2-yl]-10,13-dimethyl-2,3,4,5,6,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[*a*]phenanthren-3-ol	CNX-14	37.42	0.75
$C_{15}H_{10}O_5$	Baicalein	CNX-15	33.52	0.21
$C_{21}H_{18}O_{11}$	Baicalin	CNX-16	40.12	0.75
$C_{20}H_{18}NO_4^+$	Epiberberine	CNX-17	43.09	0.78
$C_{29}H_{50}O$	BETA-SITOSTEROL	CNX-18	36.91	0.75
$C_{25}H_{22}O_5$	*cis*-and-trans-Inophyllolide	CNX-19	38.81	0.85
$C_{15}H_{10}O_6$	Kaempferol	CNX-20	41.88	0.24
$C_{29}H_{48}O$	alpha-Spinasterol	CNX-21	42.98	0.76
$C_{29}H_{48}O$	Stigmasterol	CNX-22	43.83	0.76
$C_{21}H_{22}NO_4^+$	Palmatine	CNX-23	64.6	0.65

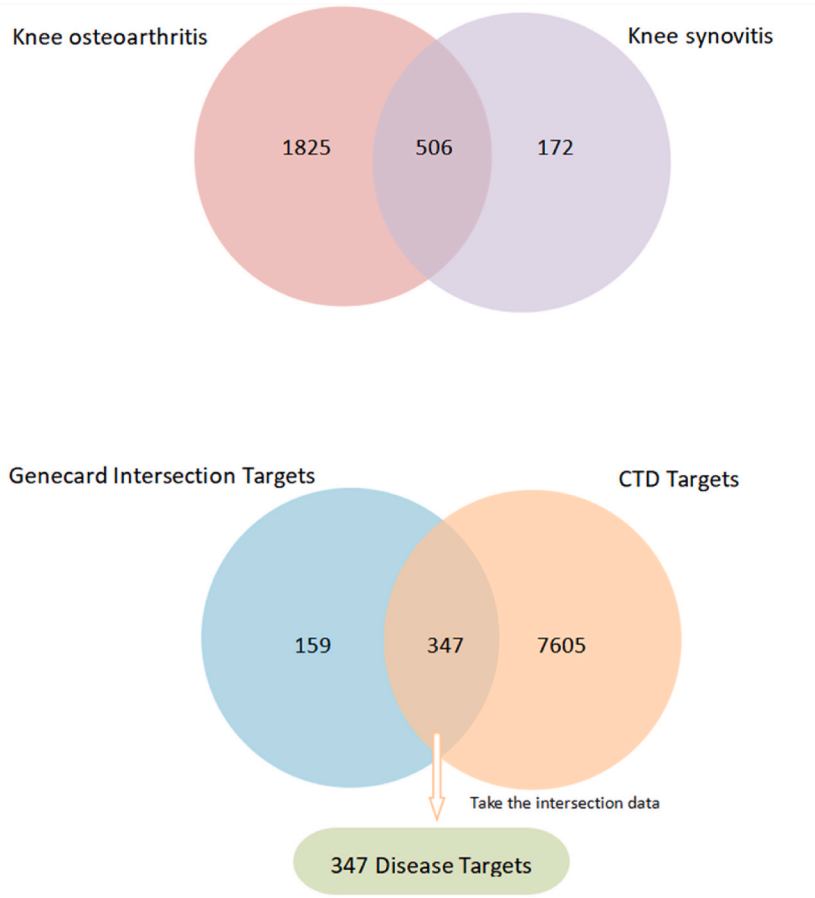

Fig. 2. Screening of CNX targets. Venn diagram of targets for CNX from GeneCards-KOA (green), GeneCards-knee synovitis (blue), GeneCards-intersection targets (yellow), CTD-KOA (cyan) databases.

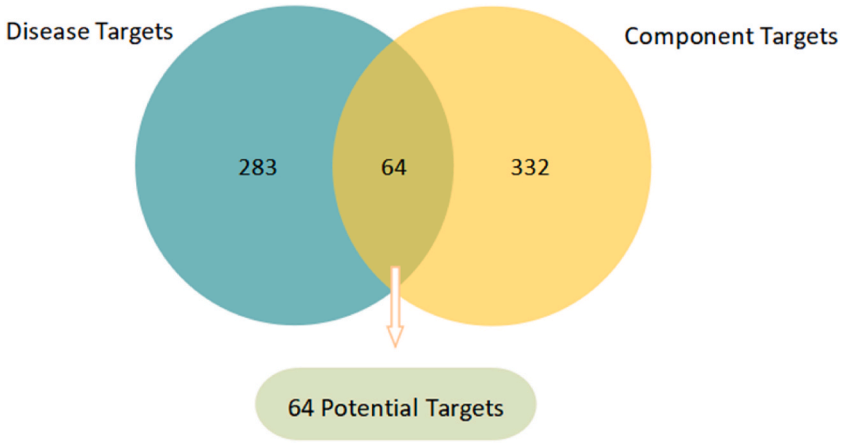

Fig. 3. A Venn diagram illustrating the potential genes in CNX and KOA. The genes that overlap are considered core genes and are represented in orange. The blue portion of the diagram represents unique drug targets, while the green portion represents unique disease targets.

3.4. GO analysis and KEGG pathways involved in the treatment of KOA with CNX

To comprehensively investigate the biological functions and pathways related to the 64 potential targets identified in Section 3.3, we conducted GO and KEGG pathway enrichment analysis using the DAVID database. Please refer to Figs. 5 and 6 for more details. The

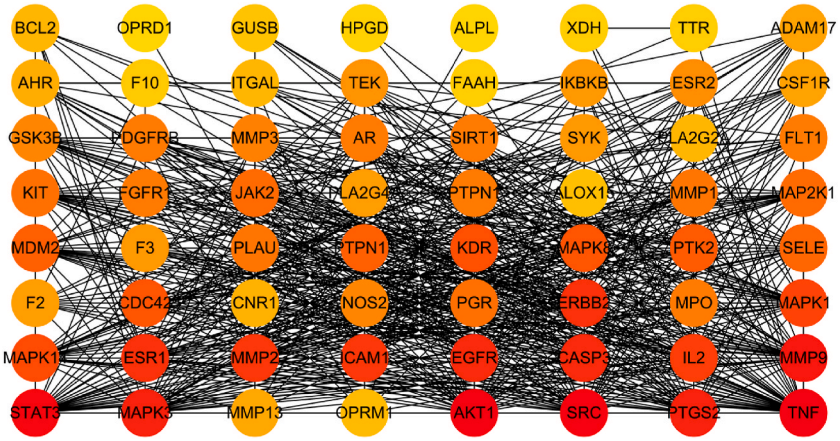

Fig. 4. PPI network construction. We identified 431 intersection genes of CNX against KOA by taking the intersection of drug targets and disease targets. These genes were then subjected to correlation analysis using STRING, resulting in the construction of a PPI visualization network. A high confidence interval of 0.4 was selected to represent the correlation between proteins. The connections in the network represent the relationships between proteins.

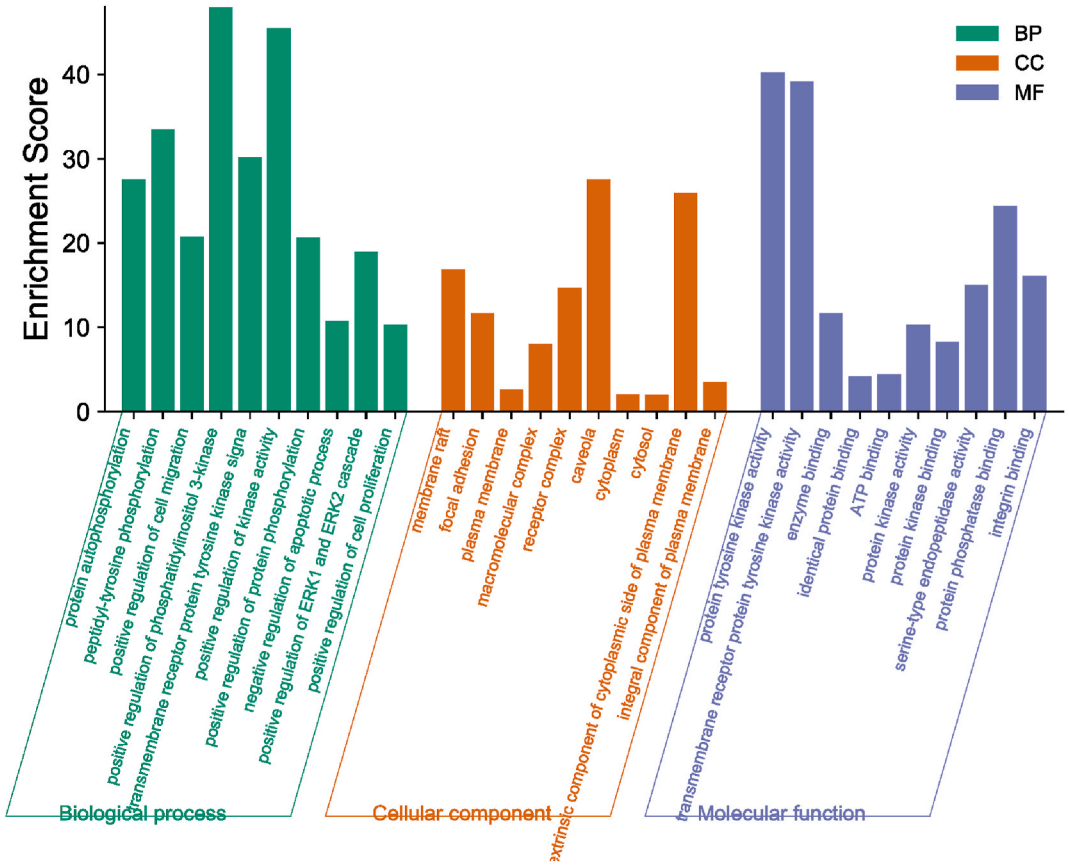

Fig. 5. Histogram of GO analyses. The DAVID database was utilized to conduct GO enrichment analyses on 64 core targets. The results were presented in a color-coded format, with green representing the biological process, orange representing the cellular component, and blue representing the molecular function.

selection of the most enriched biological processes, cellular components, molecular functions, and pathways was based on their respective p-values. The study identified several enriched biological processes, including the inflammatory response, immune response, MAPK cascade, and response to lipopolysaccharide. These enriched pathways were categorized into inflammation (e.g., NF-

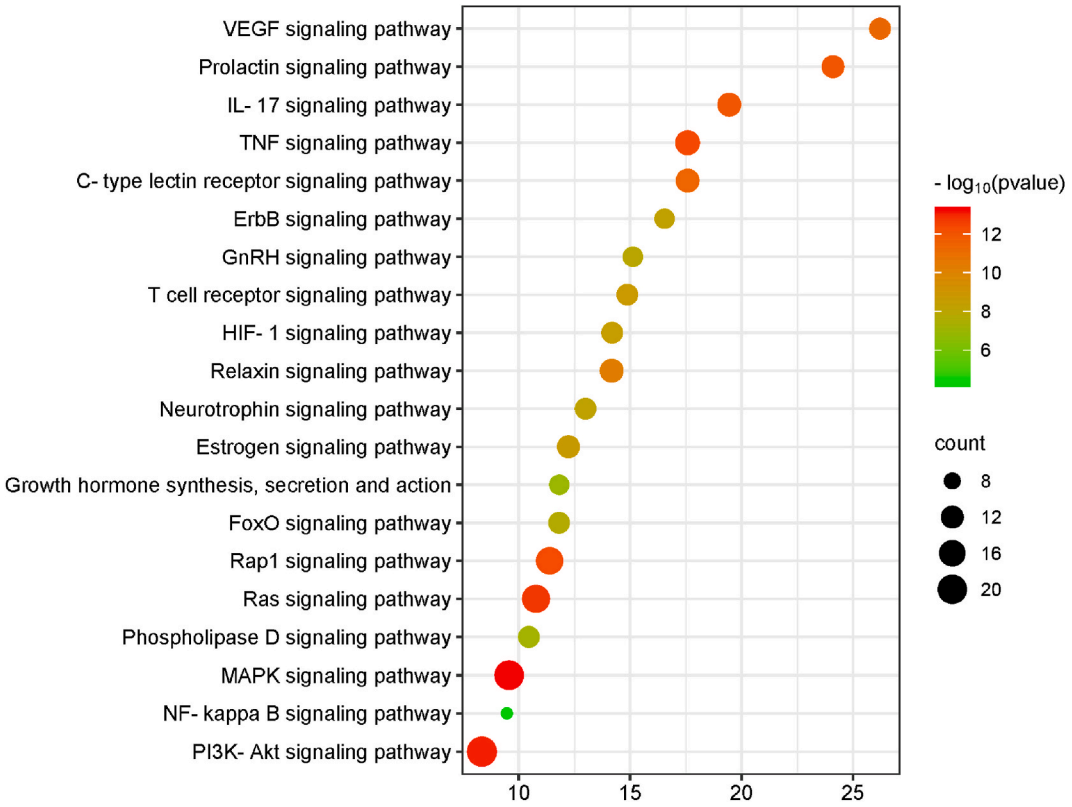

Fig. 6. Bubble diagram of functional pathway analysis. Pathway enrichment analyses of 64 core targets using the DAVID database. In the visualization, the size of the circle corresponds to the number of genes, while the shade of the color represents the significance level (p-value).

kappa B signaling pathway), immune response (e.g., T cell receptor signaling pathway), and signal transduction (e.g., MAPK signaling pathway). Additional analysis of the literature revealed a strong association between the NF-κB and MAPK signaling pathways with KOA, suggesting that they could be important targets for the treatment of KOA using CNX [21].

3.5. Ingredient-target-pathway network construction

An integrated ingredient-target-pathway network was constructed using Cytoscape 3.9.1. The network, shown in Fig. 7, comprised of 58 nodes and 306 edges. Vital targets were identified based on higher degrees of connectivity. These targets were found to closely interact with the NF-κB signaling pathway and MAPK signaling pathway. Among the 6 identified targets (IKBKB, NFKB1, RELA, MAPK1, MAPK8, and MAPK14), they may play important roles in these two pathways. This further emphasizes the significance of these pathways in the treatment of KOA by CNX.

3.6. Verification of the effect of CNX on KOA

3.6.1. Effect of CNX on the viability of chondrocyte cells

To investigate the impact of CNX on chondrocyte viability and determine the optimal intervention concentration, as well as its effect on IL-1β-induced chondrocyte viability, we employed the CCK-8 method. Our CCK-8 experiments revealed that the viability of chondrocytes remained unaffected at CNX concentrations ranging from 0.01 to 0.1 μg/mL. However, viability decreased when the concentration was increased to 1–100 μg/mL (Fig. 8 A). Furthermore, when chondrocytes were treated with IL-1β, their viability decreased, but there was no significant difference observed between the CNX group and the control group (Fig. 8 B).

3.6.2. Effect of CNX on inflammatory factors and key gene mRNA expression levels

The role of the MAPK pathway and NF-κB pathway in inflammation and immune regulation is crucial in the treatment of KOA with CNX. This study focused on examining the mRNA expression of key genes involved in these pathways, including IKBKB, NFKB1, RELA (associated with the NF-κB pathway), MAPK1, MAPK8, MAPK14 (associated with the MAPK pathway), and IL-6 (related to the inflammatory response). The expression of these genes in chondrocytes was detected using qPCR after inducing IL-1β.

In terms of inflammation-related gene expression, the cells treated with IL-1β showed a significant upregulation of inflammatory cytokines, particularly IL-6, compared to the control group ($p < 0.001$). However, the release of these cytokines was noticeably

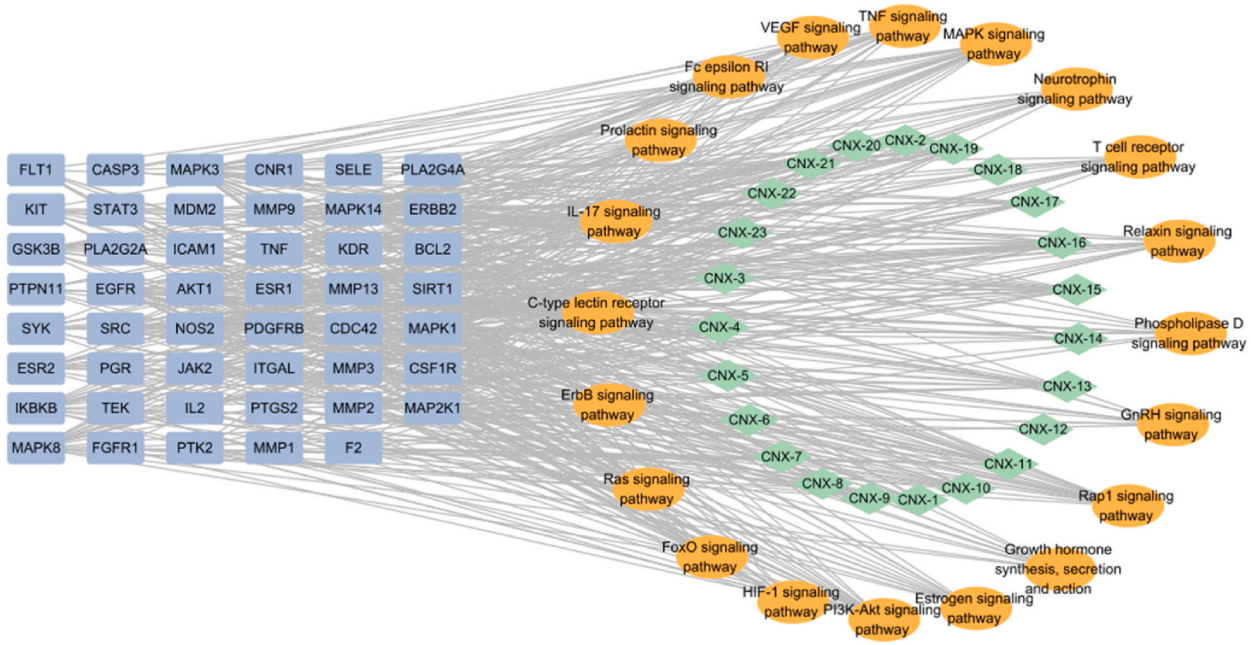

Fig. 7. Ingredient-target-pathway network. We analyze the relationships among the main ingredients, potential targets, and the top 20 pathways. In our analysis, the ingredients in CNX are represented by green rhombus nodes, the potential targets are represented by blue nodes, and the top pathways are represented by orange nodes.

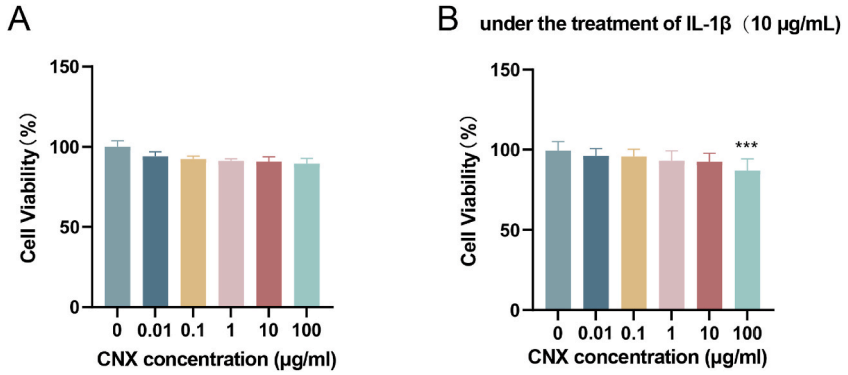

Fig. 8. The effect of CNX on cell viability under blank condition (A) and IL-1β (10 μg/mL) condition (B) To induce chondrocyte degeneration, the cells were stimulated with IL-1β. Control chondrocytes were incubated with the same dose of DMSO as the CNX group and cultured for 24 h. Subsequently, the cells were treated with different concentrations of CNX (0.01, 0.1, 1, 10 and 100 μg/mL) for 24 h, and cell viability was assessed using the CCK-8 method. Additionally, chondrocyte cells were incubated with IL-1β (10 μg/mL) and various concentrations of CNX (0.01, 0.1, 1, 10 and 100 μg/mL) for 24 h. The results were compared to the control group and the model group, which was treated with IL-1β alone (n = 10).

reduced after treatment with CNX ($p < 0.01$) (Fig. 9 G).

In terms of key genes, the qPCR analysis revealed that the mRNA expression levels of key genes associated with the MAPK and NF-κB pathways were significantly affected by CNX treatment in chondrocytes stimulated with IL-1β. Compared to the control group, the mRNA expression levels of MAPK1, MAPK8, and MAPK14 were significantly elevated in chondrocytes stimulated with IL-1β ($p < 0.01$). After treatment with CNX, the mRNA expression levels of these genes showed a significant reduction ($p < 0.05$) (Fig. 9 A, B and C). Similarly, in chondrocytes stimulated with IL-1β, the mRNA expression levels of NFKB1 and RELA increased, while IKBKB significantly reduced ($p < 0.01$). However, CNX treatment attenuated the increase in NF-κB and RELA expression, as well as the reduction in IKBKB expression (Fig. 9 D, E, F). Furthermore, CNX treatment led to an increase in the mRNA expression level of IKBKB, suggesting that CNX may inhibit the activation of NF-κB by promoting the expression of IKBKB, a known inhibitor of NF-κB.

Network pharmacology analysis revealed that both the MAPK signaling pathway and the NF-κB signaling pathway play crucial roles in the treatment of KOA by CNX. The genes highlighted in orange signify the key genes within these pathways, while the genes in green represent other genes associated with these pathways, as shown in Fig. 10.

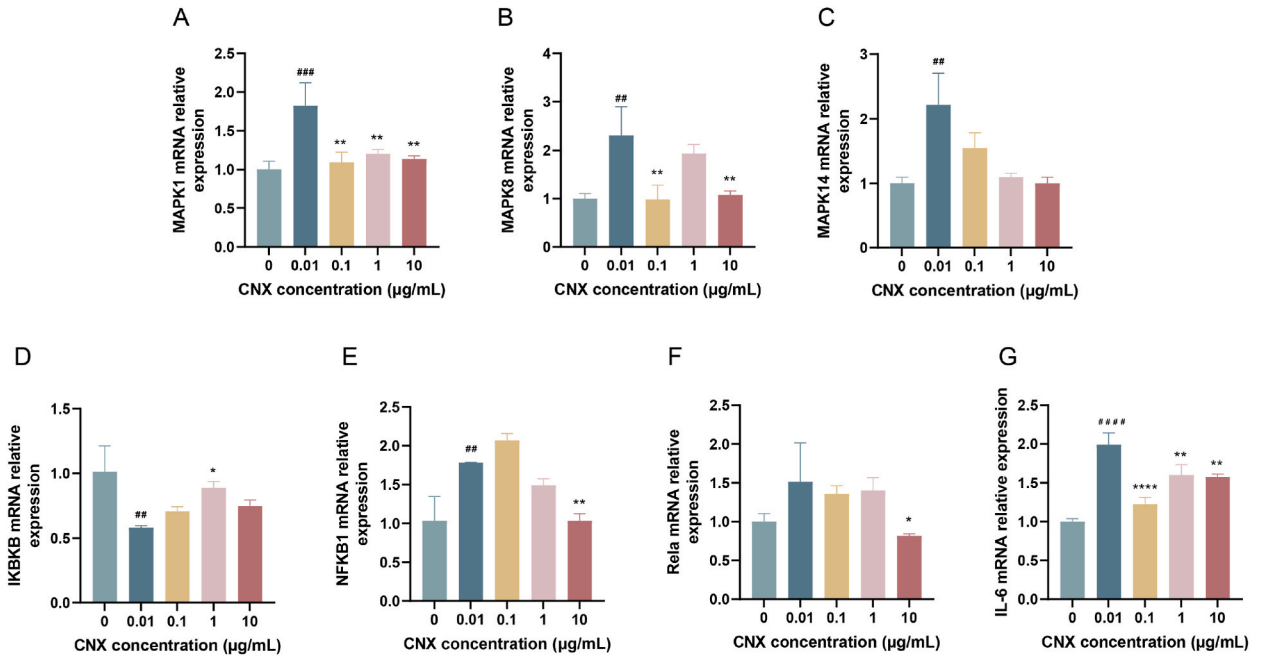

Fig. 9. The effect of CNX on the mRNA relative expression of MAPK1 (A), MAPK8 (B), MAPK14 (C), IKBKB (D), NFKB1 (E), RELA (F) and IL-6 (G) using qPCR. Control chondrocytes were treated with DMSO (the same dose as CNX) for 24 h, while different concentrations of CNX (0.01, 0.1, 1, 10 μg/mL) were added to chondrocytes incubated with IL-1β (10 μg/mL) for 24 h. The mRNA expression of MAPK1, MAPK8, MAPK14, IKBKB, NFKB1, RELA and IL-6 was measured in different groups ($n = 3$).

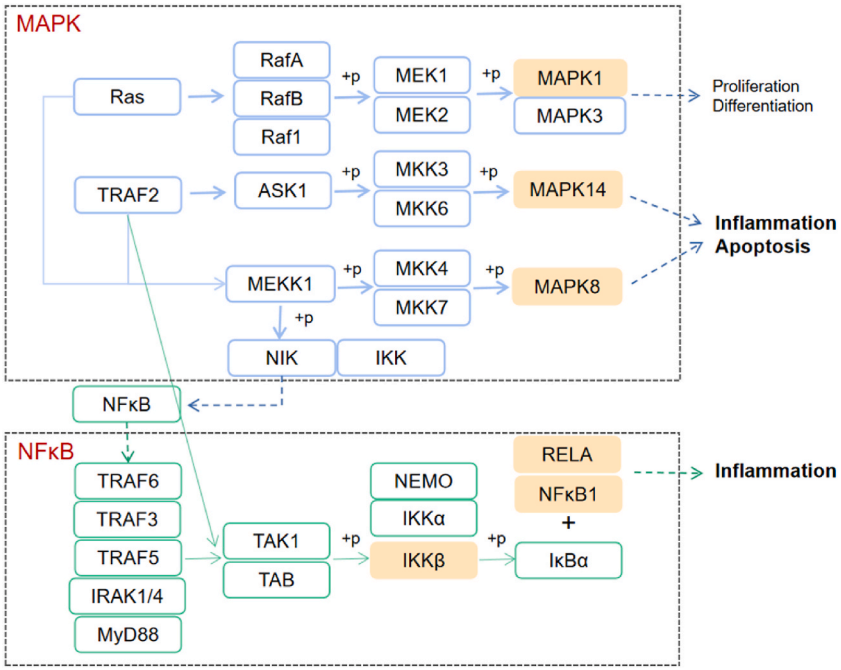

Fig. 10. Diagram of major pathways of signaling pathways.

These findings indicate that CNX may play a role in regulating the inflammatory and immune response in KOA by modulating the MAPK and NF-κB pathways.

4. Discussion

Knee osteoarthritis (KOA) is a common joint disease that causes knee joint pain and limited mobility, mainly due to external trauma or accumulated cartilage degeneration [22,23]. Currently, commonly used clinical treatments include non-steroidal anti-inflammatory drugs, corticosteroid injections, and tramadol to relieve symptoms [11]. However, these treatments only have a weak effect on pain relief and may increase the risk of cardiovascular disease [24]. Regenerative medicine and surgical arthroplasty are also becoming increasingly popular, but the sample size of scientific studies and clinical data is still insufficient to support and explain the efficacy and mechanisms of regenerative medicine, and arthroplasty is time-sensitive and not guaranteed to be effective in the long term [8,25]. Although significant progress has been made in the study of KOA in recent years, its mechanism of occurrence and development is still unclear.

In Chinese medicine, CNX has been used to dispel wind and remove blood stasis, strengthen bones and kidneys, and is widely used to treat fall injuries, rheumatic joint pain, foot laxity, and muscle contractures7. Chinese herbal tonics and proprietary Chinese medicines containing CNX are often used in clinical practice to treat various orthopedic diseases (e.g., bone injury, osteoarthritis, rheumatoid arthritis, arthralgia), gynecological diseases, and urological diseases [12]. In addition, there are experimental results showing that the monomeric component of CNX helps to alleviate the degradation of cartilage matrix and increase the expression of type II collagen mRNA in chondrocytes [26]. Although CNX is currently regarded as an effective and key herbal medicine for the treatment of KOA [27], existing studies have mostly focused on the effects of CNX's individual components, while research on the overall mechanism of action and targets of CNX's single drug remains limited, hindering the further development of the drug.

To investigate the potential targets and pathways affected by CNX in the treatment of KOA, this study employed network pharmacology. The relevant pathways and targets were predicted and then validated using an IL-1β-induced apoptosis assay in vitro. A comprehensive search of the TCMSP database yielded 23 major chemical components of CNX. SwissTarget Prediction was utilized to predict the target of CNX components. KOA disease targets were obtained from Genecards and CTD. After mapping 396 CNX target genes with 283 KOA-related genes, a total of 64 intersection target genes were identified. These target genes represent potential points of intervention for CNX treatment of KOA. A.

PPI network was created using the 64 target genes. Following GO functional and KEGG pathway enrichment analysis, it was observed that the MAPK and NF-κB signaling pathways were significantly enriched. These pathways were primarily associated with six specific targets: MAPK1, MAPK8, MAPK14, IKBKB, NFκB1, and Rela.

The NF-κB pathway plays a crucial role in regulating and influencing a wide range of biological processes, including cell survival, proliferation, differentiation, apoptosis, aging, inflammation, and immune responses [28]. Activation of the NF-κB pathway is necessary for the activation of signal cascades involving MMP proteins, inducible nitric oxide synthase (iNOS), IL-1β, TNF-α, and other cytokines [29]. In this study, IL-1β, as an initiator of the inflammatory signaling pathway NF-κB, significantly increased the IL-6 content in chondrocytes after induction. The results after CNX intervention showed that CNX can reduce the content of NFκB1 and Rela while increasing the expression of IKBKB. It has been further shown that activation of the NF-κB pathway produces a cascade response to the MAPK pathway [30]. Growth factors or inflammatory stimuli released as a result of NF-κB pathway activation are delivered from the extracellular space to the nucleus [31], driving the expression of downstream genes, such as MMPs, and ultimately accelerating the onset of KOA.

The MAPK pathway can be activated not only by the cytokines produced by the NF-κB pathway to accelerate the formation of KOA, but also by the inflammatory cartilage matrix itself. This activation leads to the degradation of typeIIcollagen and directly accelerates the degeneration and apoptosis of chondrocytes [2]. Fan et al.'s study indicated that the phosphorylation level of p38 in the articular cartilage of KOA patients was significantly higher than that in normal articular cartilage tissue [32]. This study's results demonstrate that CNX intervention reduces the level of cell apoptosis after IL-1β induced degeneration of chondrocytes. CNX can significantly inhibit the generation of MAPK1, MAPK8, and MAPK14 in the MAPK pathway, partially blocking the MAPK signaling pathway and inhibiting the inflammatory response.

In conclusion, network pharmacology and in vitro assays indicate CNX's potential to inhibit the MAPK and NF-κB pathways, with key targets including MAPK1, MAPK8, MAPK14, IKBKB, NFκB1, and Rela, thereby modulating chondrocytic inflammatory responses. Further research is warranted to elucidate the extent of CNX's pathway blockade.

CRediT authorship contribution statement

Zhicheng Yao: Writing – original draft, Methodology. **Fengping Gan:** Writing – original draft, Visualization. **Yuqing Zeng:** Software. **Litong Ren:** Methodology. **Yirong Zeng:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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