Target prediction of anti-chronic liver injury of Kangxian pill based on network pharmacology

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Research

Keywords: Kangxian Pill, network pharmacology, chronic hepatic injury, PI3K/Akt signaling pathway, apoptosis, inflammation

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Target prediction of anti-chronic liver injury of Kangxian pill based on network pharmacology

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Abstract

Background: The present study aimed to validate the protect effect of Kangxian pill (KXP) on chronic hepatic injury (CHI) and investigate its potential mechanism by network pharmacology-based prediction and experimental verification in vivo.

Methods: The effect of KXP in the treatment of carbon tetrachloride (CCL4)-induced CHI is investigated by calculating liver index, measuring AST and ALT levels and performing HE staining. Targets of active ingredients of KXP were predicted in TCSMP and targets of chronic liver injury were searched in DisGeNET, OMIM and GeneCards databases. We obtain some pivotal targets of KXP for the treatment of CHI by intersecting the targets of KXP and CHI. Subsequently, we performed gene ontology (GO) functional and pathways enrichment analyses, as well as conducted networks based on potential targets to determine the core targets and representative pathways. We further validated expressions of IL-6, IL-1β, TNF-α, Bax, Bcl2, PI3K, Akt, and p-Akt according to the potential molecular mechanisms analyzed based on network pharmacology analysis.

Results: The results showed that the levels of AST and ALT in serum decreased after treatment with KXP. HE staining also revealed that KXP could improve hepatocyte abnormality in vivo. A total of 81 potential targets of KXP in the treatment of CHI were identified through network pharmacology analysis. After integrating potential targets, function enrichment, representative pathways and networks, we identified PI3K, AKT1, BCL2, TNF-α, IL-1β, and IL-6 as potential targets, which may play a vital role in the KXP treatment. The experimental results also showed that KXP could down-regulate the mRNA and protein expression of IL-1β, IL-6, TNF-α and Bax, and up-regulate the PI3K and p-Akt protein expression in vivo.

Conclusions: Our results suggest that KXP could alleviate CHI through regulating inflammation and apoptosis and provide deep insight into the hepato-protective mechanisms.

Key words: Kangxian Pill, network pharmacology, chronic hepatic injury, PI3K/Akt signaling pathway, apoptosis, inflammation
1. Introduction

Chronic hepatic injury (CHI), characterized by elevated aminotransferase levels, excessive inflammatory response, hepatocyte apoptosis/necrosis, irregular hepatocyte regeneration, and fibrous tissue hyperplasia, could be caused by viral infections, drugs, alcohol, chemical agents and biological factors as well as auto-immune hepatitis. Although there has been remarkable progress in treatment of CHI over the last several decades, most of the therapies still fail to completely cure patients due to the complex pathogenesis of CHI, leading to recurrent and persistent liver injury and eventually causing liver fibrosis, cirrhosis and even liver cancer. Therefore, it is urgent to develop candidate medicines against CHI and to further determine molecular mechanisms that involved.

Over recent decades, an increasing number of traditional Chinese medicines (TCMs) have been widely applied to treat CHI worldwide due to the high abundance, pharmacological activity, chemical diversity and low side effects. In theory, TCM formulas might act on multi-targets and regulate multi-signaling pathways simultaneously. In clinical practice, the use of TCM formulas could enhance the efficacy and mitigate side effects, and synergistically improve the homeostasis of the body, exhibiting superior overall therapeutic effect over single compounds. Kangxian pill (KXP), an empirical formula of Tianjin Second People’s Hospital, is composed of 12 kinds of TCMs, including Angelica sinensis, Ligusticum chuanxiong hort, Scutellaria baicalensis Georgi, Astragalus membranaceus (Fisch.) Bunge, Radix Pseudostellariae and seven others. KXP has been applied to control the progression of liver diseases for over 40 years with remarkable efficacy. Our previous study showed that KXP exerted hepatic protective effect through decreasing levels of transaminase and raising activities of superoxide dismutase (SOD) and increasing contents of and glycogen (Gn). In addition, KXP could alleviate chronic liver injury through regulating gut microbiota to inhibit permeability of intestinal epithelial. However, major hepato-protective targets of KXP remain unclear.

Network pharmacology provides a novel method to further study the relationship between medicines and diseases by integrating system biology, multi-directional pharmacology, bioinformatics, and computer science. The main compounds and potential targets of TCMs protect against diseases could be studied at the systemic level by using network pharmacology, which contributes to explain the multi-component and multi-target characteristics of TCMs. Therefore, using network pharmacology approach to study compounds, herbals and formulas could facilitate its transformation from a single-target to a multi-component-target network. In the current study, we combined network pharmacology and *in vivo* experiments to explore the potential targets and pathways of KXP to protect against CHI.

2. Materials and Methods

2.1 Reagents and chemicals

Kangxian Pill (KXP) was provided by Tianjin Second People's hospital. Diammonium glycyrrhizinate (DG) was purchased from Chia Tai Tianqing Pharmaceutical Group Co., Ltd. (Jiangsu, China). CCL$_4$ (≥99% purity) was purchased...
from Tianjin Kaitong Chemical Reagent Co., Ltd (Tianjin, China). Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) Assay Kits were supplied from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA Kits of Mouse Interleukin1β (IL-1β), Mouse Interleukin 6 (IL-6) and Mouse Tumor Necrosis Factor α (TNF-α) were purchased from Wuhan Genmei Biotechnology Co. LTD (Wuhan, China). RNAsimple Total RNA Kit, FastQuant RT Kit and SuperReal PreMix Plus were purchased from TIANGEN BIOTECH (BEIJING) CO., LTD (Beijing, China). The antibodies against phosphoinositide 3-kinase (PI3K), p-Akt: phosphorylated (p-Akt), and protein kinase B (Akt) were purchased from Cell Signaling Technology, Inc. (Massachusetts, USA). The antibodies against Bax, Bcl2 and glyceraldehyde phosphate dehydrogenase (GAPDH) were supplied from Abcam plc. (Cambridge, UK).

2.2 Preparation of KXP

KXP consists of 12 TCMs, including Angelicae Sinensis Radix, Ligusticum chuanxiong Hort., Radix Paeoniae Rubra, Cordyceps, Scutellariae Radix, Trionycis Carapax, Toosendan Fructus, Spreading Hedyotis Herba, Astragali Radix, Pseudostellariae Radix, Schisandraceae Chinensis Fructus, Glycyrrhizae Radix Et Rhizom, which were purchased from Tianjin traditional Chinese Medicine prepared pieces Co., Ltd. (Tianjin, China). All medicinal slices were appraised by Pharmacist Li Wang of the Tianjin Second People’s Hospital and then weighed, pulverized and mixed in proportion to acquire KXP.

2.3 Network Pharmacology-based prediction of KXP protect against CHI

2.3.1 Identification of components and targets of KXP

The oral bioavailability (OB) ≥ 30% and drug likeness (DL) ≥ 0.18 were selected as screening conditions, all active components of KXP were collected from Compound Reference Database Chinese Academy of Sciences (http://www.chemcpd.csdb.cn/cmpref/default.asp) and Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) (http://tcmspw.com/tcmsp.php). Targets associated with the selected bioactive components were obtained from TCMSP. Then, all targets information were standardized in UniProt (https://www.uniprot.org/) database after deleting duplicate values.

2.3.2 Potential targets Interaction of KXP with CHI

In order to collect the disease targets, we searched in DisGeNET database (https://www.disgenet.org/home/) using the keyword ‘chemically-induced liver toxicity’ as well as searched in Online Mendelian Inheritance in Man (OMIM) database (https://www.omim.org/) and GeneCards database (https://www.genecards.org/) with the keyword “chronic hepatic injury (CHI)”. In the Venny database (https://bioinfopg.cnb.csic.es/tools/venny/index.html), potential targets of KXP in the treatment of CHI were obtained intersection of disease targets and medicines targets.

2.3.3 Enrichment analysis and Network construction for potential targets
The GO function enrichment analysis, and representative pathways related to KXP in the treatment of CHI were analyzed using the Metascape database (https://www.plob.org/tag/metascape). Visualization of biological processes and representative signaling pathways associated with potential targets of KXP treatment on CHI were organized and imported by GraphPad Prism and bioinformatics platform (http://www.bioinformatics.com.cn/) independently. Networks of Component-target (C-T), Protein-protein interaction (PPI) and Target-Pathway (T-P) were performed using Cytoscape 3.7.2.

2.4. Experimental Verification
2.4.1 Mice and experimental design
BALB/c mice (male, 6-8 weeks old, weighted 20g) were provided by SPF (Beijing) Biotechnology Co., Ltd. (Animal license number: SCXK (JING) 2019-0010). All mice were fed in a specific pathogen-free condition in the environment of temperature (24±2℃) and humidity (50±10%). After keeping under the standard condition for a week, the model mice (n=30) were treated with 2ml/kg of CCL₄ diluted (1:4 v/v) in olive oil by intraperitoneal (i.p.) injections, except the normal mice (the Control group n=10) were treated with 2ml/kg of olive, twice a week for 6 weeks. After the models were built, all model mice were randomly divided into three groups (n=10): Model group, DG group (positive group) and KXP group. The Control group and Model group were treated with saline solution (10ml/kg), the DG group were treated with DG (60mg/kg) and the KXP group were treated with KXP (3g/kg) by intragastric administration for 4 weeks. At the end of experiment, mice were euthanasia and then serum samples and liver tissues were collected for further assays.

2.4.2 Liver index calculation
All mice were weighted and euthanized at the end of experiment. After liver tissues were collected, washed, dried with filter paper and then weighted, the liver indices were calculated. The formula is as follows: Liver index=liver weight(g)/body weight(g) × 100% ¹¹.

2.4.3 Biochemical assays
The activities of ALT and AST in serum were assayed according to experimental steps of manufacturer’s instructions. Pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, were analyzed according to the manufacturer’s instructions by ELISA. Absorbance at 450 nm was determined using a Micro plate reader.

2.4.4 HE staining
The liver tissues with 1cm³ were cut to fix in 10% neutral buffered formalin solution. The liver samples were dehydrated in graded ethanol (50%, 70%, 85%, 95%, 100%), cleared in xylene and embedded in paraffin. Sections of 5μm were cut and stained with Hematoxylin-Eosin (HE). Pathological changes of livers were observed using a light microscope.
2.4.5 Quantitative real-time PCR

Total RNA was extracted from liver samples using Trzol, then, the mRNA was converted into cDNA for carrying out quantitative real-time PCR (qPCR). The procedure qPCR was performed for one cycle of 95°C for 15min, 40 cycles of 95°C for 15s and 60 °C for 30s. The housekeeping gene GAPDH was used for normalization. The fold changes were calculated using the method of $2^{-ΔΔCt}$. All primer sequences used in this study have been shown in Table1.

| Genes | Primer sequence(5'-3') |
|-------|-----------------------|
| GAPDH | F: 5'-AACGACCCCTTCATTGACCT-3’ R: 5’-TGGAAGATGGTGATGGCCTT-3’ |
| Bcl2  | F: 5'-TGCGAGAGATGATTGCTGAC-3’ R: 5’-GATCAGCTCGGCACCTTAG-3’ |
| Bax   | F: 5’-TGCGAGAGATGATTGCTGAC-3’ R: 5’-GATCAGCTCGGCACCTTAG-3’ |
| IL-6  | F: 5’-CTGCAAGAGACTTTCACTCCAGC-3’ R:5’-AGTGGTATAAGCAGGTCTGTTTG-3’ |
| IL-1β | F: 5’-GAAATGCCACCTTTTGACAGTG-3’ R: 5’-TGGA GTCTCCTCATCAGGACAG-3’ |
| TNF-α | F: 5’-CACAGAAAGCAGATCCGC-3’ R: 5’-TAGACAAGAGCCTTGGTGG-3’ |

2.4.6 Western Blot

The total protein was extracted using RIPA and PMSF (PMSF: RIPA=1:100) and then protein concentration was detected. Equivalent amounts of protein (20μg) were loaded and separated on SDS-PAGE gel electrophoresis and then transferred to Polyvinylidene Fluoride (PVDF) membranes. The membranes were incubated with primary antibodies at 4 °C overnight after blocking in 5% non-fat milk for 2h at room temperature. The primary antibodies were rabbit polyclonal antibodies against PI3K (1:1000 dilution), Akt (1:1000 dilution), p-Akt (1:1000 dilution), Bax (1:5000 dilution) and Bcl2 (1:1000 dilution). All membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (1:10000 dilution) for 1h at room temperature before being visualized by using ECL luminescence reagents. Loading was normalized with GAPDH (1:10000 dilution). All experiments were performed three times independently and grayscale value of bands were analyzed using Image J.

3. Statistical analysis

Statistical analyses were analyzed using Statistical Package for the Social Sciences (SPSS, Version 17.0.) expressing as the mean± standard deviation (SD). The differences between experimental groups were compared using Student’s t-test and one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. Drawings were conducted using GraphPad Prism 7.

4. Results

4.1 KXP ameliorated CCL4-induced hepatic injury

To determine whether KXP could treat CHI in vivo, we firstly induced liver injury by CCL4 and then treated with KXP. Compared to the Control group, the liver index and AST and ALT levels in serum increased obviously in the Model group. In contrast, liver indexes and levels of AST and ALT decreased significantly in the DG and KXP
groups compared with the model group (Fig 1A, 1B, 1C). The result of HE staining showed that normal liver cells were clear and liver lobules structure were normal in the Control group. However, the hepatic lobules structure disordered in the model group, with extensive edema and partial necrosis of hepatocytes. Treatment with DG or KXP could significantly improve the histopathological changes (Fig 1D.). These results indicated that KXP could alleviate chronic liver injury induced by CCL4.

Fig. 1. Effect of KXP on CCL4-induced CHI. A. Liver indices of all groups. B. The level of AST in serum. C. The level of ALT in serum. D. HE staining of liver tissues (20×). Results were represented as mean±SD. n=10. *p < 0.01, vs. the Control group, **p < 0.01, vs. the Model group. Scale bars, 100μm. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.

4.2 Network Construction

KXP consists of 12 TCMs, which play an important role in treating CHI through multi-targets and multi-pathways. To explain these complicated relationships, function enrichment analysis and networks were conducted based on network pharmacology.

4.2.1 Drug C-T Network Analysis.

A total of 385 targets of KXP were obtained from TCMSP after deleting the duplicate targets. In addition, 1088 candidate targets related to CHI were collected from DisGeNET, OMIM and GeneCards. In order to intuitively obtain comprehensive understanding of the relationship between components and their targets, the C-T network was conducted using Cytoscape 3.7.2 (Fig. 2) after 165 targets were determined as pivotal therapeutic targets of KXP in the treatment of CHI from the Venn figure (Fig. 3A). In the C-T network, it included 327 nodes (12 for drugs, 150 for bioactive components and 165 for potential targets).
4.2.2 PPI network analysis

STRING database provides information on experimental and predicted interactions. We uploaded 165 common targets into STRING database to obtain the interaction information, which was imported into Cytoscape 3.7.2 to conducted PPI network. The magnitude of ‘Degree’ is proportional to its role in the central correlation. Therefore, the median of ‘Degree’ was used as a screening condition. A total of 81 potential targets were screened for conducting the PPI network according to the median of ‘Degree’ greater than 33 (Fig. 3 B). The top 10 genes of the PPI network, ranked by degree value, including TP53, JUN, CASP3, AKT1, VEGFA, IL6, STAT3, MAPK8, MAPK3, and TNF.

**Fig. 2.** The C-T network of potential targets. The blue rhombus represent potential targets, purple squares represent drugs and other hexagons represent active ingredients. In addition, nodes’ size are proportional to their ‘degree’.

**Fig. 3.** Venn chart and PPI network of potential targets. A. Venn chart conducted using Venny database. B. PPI network of 81 potential targets performed in Cytoscape 3.7.2. The size of the nodes is proportional to the value of the ‘Degree’.
4.2.3 GO functional enrichment analysis

A total of 81 potential targets were uploaded into Metascape database to obtain GO functional enrichment analysis, including biological process (BP), cellular component (CC) and molecular function (MF), which could illustrate the involved biological functions. The top 12 significantly enriched terms were selected for visualization according to the P values of potential targets enriched in BP, CC and MF, which indicates that KXP could regulate cell apoptosis, oxidative stress via cytokine receptor, protein kinase, protein phosphatase binding to membrane raft/region and serine/threonine protein kinase complex exerting cure effects against CHI (Fig. 4A). Overall, these biological processes were mostly related to oxidative, inflammatory response and apoptosis.

4.2.4 Pathway enrichment analysis

Enrichment analysis of 81 potential targets predicted representative signaling pathways for KXP in treatment of CHI. The top 30 significantly enriched representative pathways related to CHI were selected for graphing. Among these representative pathways, the PI3K/ Akt signaling pathway is the most relevant pathway for KXP in the treatment of CHI based on the counts of potential targets. In addition, apoptosis, IL-17 and NF-κB signaling pathways were also included (Fig 4B).

4.2.5 T-P network construction

It is generally accepted that genes cannot illuminate their biological and pharmacological activities independently. Therefore, we performed the T-P network based on the top 30 signaling pathways and related targets to further illustrate the molecular mechanism of KXP in the treatment of CHI (Fig 5). We identified PI3K, AKT1, BCL2, IL-1β, IL-6 and TNF-α as considerably high-relevant targets in apoptosis and inflammation after integrating potential targets and analyzing function enrichment and pathways as well as conducting networks. Therefore, we supposed that the anti-hepatic injury of KXP could inhibit apoptosis and inflammation by regulating PI3K/Akt signaling pathway.

Fig. 4. The GO and representative pathways enrichment analysis. A. GO enrichment analysis of 81 potential targets conducted using GraphPad Prism. The green bars represent BP, the purple bars represent CC, and the blue bars represent MF. B. Representative pathways enrichment analysis of 81 potential targets.
CC and the orange bars represent MF. B. The representative pathway enrichment analysis of 81 core targets conducted using GraphPad Prism. The node size represents gene counts and the colors of nodes from red to green illustrate the $P$ value from small to more.

Fig. 5. The Target-Pathway (T-P) network. The purple squares represent the potential genes and the red squares represent the signaling pathways. The node size indicates the magnitude of ‘Degree’.

4.3 Experimental Validation

To explore whether these potential targets predicted by network pharmacology play important roles in KXP protect against CHI. We further validated the anti-inflammatory and anti-apoptotic effects of KXP by in vivo experiment.

4.3.1 KXP could inhibit CCL$_4$-induced inflammation response

IL-6, IL-1$\beta$ and TNF-$\alpha$ are major pro-inflammation, that mediate liver injury. In the present study, compared with the Control group, IL-6, IL-1$\beta$ and TNF-$\alpha$ mRNA expression increased significantly in the Model mice, but expression of these pro-inflammation cytokines decreased in the DG and KXP group (Fig 6A, 6B, 6C). Same as the gene expression, the secretion levels of IL-6, IL-1$\beta$ and TNF-$\alpha$ in peripheral blood in the Model group increased, and decreased after treatment with KXP (Fig 6D, 6E, 6F). These results suggested that KXP could inhibit the overproduction of pro-inflammation cytokines in mice with CCL$_4$-induced CHI.
Fig. 6. The effect of KXP protect against CHI by inhibiting inflammation. A. Gene expression of IL-6. B. Gene expression of IL-1β. C. Gene expression of TNF-α. D. Protein expression in serum of IL-6. E. Protein expression in serum of IL-1β. F. Protein expression in serum of TNF-α. The results were presented as mean±SD. #p < 0.05, ##p < 0.01, vs. the Control group. *p < 0.05, **p < 0.01, vs. the Model group. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.

4.3.2 KXP could regulate CCL4-induced hepatocyte apoptosis

To investigate whether KXP treatment could decrease hepatocyte apoptosis, apoptosis-associated protein Bax and Bcl2 were evaluated. These results showed that, compare with the Control group, gene and protein expression of Bax were up-regulated and the gene expression of Bcl2 was down-regulate in CCL4-induced Model group. As we expected, gene and protein expression of Bax (Fig 7A, 7C, 7D) were down-regulate and gene expression of Bcl2 (Fig 7B) was up-regulate in the DG and KXP group compared to the Model group. Although, there was no significant change of the Bcl2 protein expression in each group (Fig 7E). These data also could show that KXP had an anti-apoptotic effect.

Fig. 7. Effects of KXP protect against CHI by regulating apoptosis. A. Gene expression of Bax. B. Gene expression of Bcl2. C. Protein expression of Bax. D. Protein expression of Bcl2.
Gene expression of Bcl2. C. Protein bands of Bax and Bcl2. D. Protein expression of Bax. E. Protein expression of Bcl2. Grayscale value of the bands were analyzed using Image J. All results were represented as mean±SD. *p < 0.01, vs. the Control group. *p < 0.05. **p < 0.01, vs. the Model group. ns: no significant. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.

4.3.3 KXP could regulate PI3K/Akt signaling pathway

To investigate whether the hepato-protective effect of KXP is related to the PI3K/Akt signaling pathway, protein expression of PI3K, Phosphorylated Akt (p-Akt) and Akt were regulated by Western Blot. Compared with the Control group, we found that protein expression of PI3K, p-Akt, and p-Akt/Akt were down-regulated in the Model group. After treatment with DG or KXP, PI3K, p-Akt, and p-Akt/Akt protein expression were obviously up-regulated compared to the Model group (Fig 8). These results indicated that KXP existed the protective effect through activating PI3K/Akt signaling pathway.

Fig. 8. The effect of KXP protect against CHI by regulating PI3K/Akt. A. Protein bands of PI3K, Akt and p-AKT. B. Protein expression of PI3K. C. Protein expression of Akt. D. Protein expression of p-Akt. E. Protein expression of p-Akt/Akt. Grayscale value of the bands were analyzed using Image J. The results was presented as mean±SD. *p < 0.05, vs. the Control group. *p < 0.05. **p < 0.01, vs. the Model group. ns: no significant. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.

5. Discussion

Several reports revealed that the pathological onsets of CHI were found to be involved in oxidative stress, lipid peroxidation, mitochondrial damage, inflammation and apoptosis. As a commonly used animal model, the mice/rats model of CCL4-induced CHI were characterized by increasing the activities of both ALT and AST as well as the extensive apoptosis/necrosis of hepatocytes. Our report demonstrated that levels of transaminase in serum of CCL4-induced mice increased, which were attenuated by KXP. Meanwhile, pathological change of liver histological also demonstrated that KXP treatment significantly alleviate CCL4-induced hepatocytes extensive edema and necrosis. In addition, our previous study demonstrated that KXP could alleviate the inflammation response of CCL4-induced CHI by regulating gut
microbiota. However, as an empirical formula, KXP consists of 12 Chinese medicines and contains multi-compounds, and it’s tough to clarify the multi-molecular mechanisms with conventional experimental methods. Therefore, it is necessary to screen and identify the therapeutic targets of KXP protect against CHI using alternative methods.

The application of network pharmacology provides a powerful tool to study the mechanism of multi-targeting of TCMs formula. We confirmed 81 targets as pivotal therapeutic targets of KXP in the treatment of CHI according to PPI analyses. Further analysis of GO annotation and representative pathways in 81 potential therapeutic targets revealed that KXP acted at multi-targets and multi-signaling pathways. Then, we identified the central targets, including AKT1, BCL2, BAX, TNF, IL6 and IL-1β. This suggests that KXP could alleviate CHI by inhibiting inflammation and apoptosis. In addition, the highest degree is PI3K/Akt signaling pathway in the C-P network. Therefore, the findings from the present work predicated that KXP could against CHI through regulating PI3K/Akt signaling pathway.

Accumulating evidences indicated that the first phase of hepatic injury is always characterized by increased hepatocyte apoptosis. The balance between the pro-apoptotic and anti-apoptotic members of the Bcl2 family, such as Bcl2 and Bax, play a significant role in cellular damage. In our study, Bax protein expression was inhibited obviously even though the protein expression of Bcl2 was not statistically significant after treating with KXP. These results provided strong evidence that KXP inhibited hepatocyte apoptosis might be dependent on down-regulating Bax.

Some studies have confirmed that long-term hepatocytes damaged could activate hepatic Kupffer cells (KCS), and then release various pro-inflammatory cytokines, like TNF-α, IL-6 and IL-1 β. Pro-inflammatory cytokines release levels are precisely regulated by keeping the balance between the pro-inflammatory and anti-inflammatory responses, which are produced by inflammatory cells. Furthermore, compared with the Model group, the mRNA expressions in the liver and the protein levels in serum of IL-1β, TNFα and IL-6 with the treatment of KXP decreased markedly in this study. These results showed that KXP had a potential anti-inflammatory effect on mice with CCL4-induced CHI.

It’s widely acknowledged that PI3K/Akt signaling pathway participate in various physiological and pathological processes, including cell growth, proliferation, differentiation and apoptosis. Akt, a serine/threonine kinase, is the primary mediator of PI3K-initiated signaling. Akt is activated by PI3K phosphorylation, p-Akt further regulates cell apoptosis by activating or inhibiting its downstream targets such as Bcl-2 and Bax. Some studies showed that PI3K or Akt inhibitors had potential hepatotoxicity on Fas-or TNFα-induced hepatic injury. To further prove whether the PI3K/Akt signaling pathway involved apoptosis and inflammation in mice with CHI, we investigated the expression of key proteins of PI3K/Akt signaling pathway. In our study, p-Akt was inhibited following CCL4 exposure and its protein expression was significantly up-regulated after treatment with KXP for 4 weeks as well. These results indicated that KXP could reduce hepatic injury through activating the PI3K/Akt signaling pathway.
Conclusions
In conclusion, this study demonstrated that KXP exerts the therapeutic effect on CCL₄-induced CHI. The combination of network pharmacology and in vivo experiments suggests that molecular mechanisms of KXP protect against CHI may be through the regulation of PI3K/Akt signaling pathway to inhibit apoptosis and decrease inflammatory response.

Abbreviations
ALT: Alanine aminotransferase, Akt: Protein kinase B, AST, Aspartate aminotransferase, BP: Biological process, CC: Cellular component, CCL₄: Carbon tetrachloride, CHI: Chronic hepatic injury, C-T: Component-target, DG: Diammonium Glycyrrhizinate, GAPDH: Glyceraldehyde phosphate dehydrogenase, GO: Gene ontology, HE: Hematoxylin-Eosin, IL-1β: Interleukin1β, IL-6: Interleukin 6, KXP: Kangxian pill, MF: Molecular function, PPI: Protein-protein interaction, PI3K: phosphoinositide 3-kinase, p-Akt: Phosphorylated protein kinase B, qPCR: Quantitative real-time PCR, T-P: Target-pathway, TCMs: Traditional Chinese medicines, TNF-α: Tumor Necrosis Factor α.

Declarations
Ethics approval and consent to participate
All animal experiments were performed with approval from the Animal Research Ethics Committee of the Tianjin University of Traditional Chinese Medicine (TCM-LAEC2019072).

Consent to publish
Not applicable

Availability of data and materials
The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests
All authors declare no conflict of interest.

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Authors’ contributions
This study was conceived by Yuhong Bian, Li Wang and Min Cao. Yuhong Bian, Min
Cao and Yiyang Wang contributed to the manuscript preparation and modification. Potential targets and pathways predicted using network pharmacology by Min Cao, Haizhao Liu and Xueqian Dong. The experiments were performed by Haizhao Liu, Mengxue Dong and Jida Wang. All data were analyzed by Min Cao, Yiyang Wang and YuTong Jin.

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Figure 1

Effect of ICXF on CCL4-induced CII L. A. Liver indices of all groups. B. The level of AST in serum. C. The level of ALT in serum. D. HE staining of liver tissues (20x). Results were represented as mean±SD. n=10. ##p<0.01, vs. the Control group. **p<0.01, vs. the Model group. Scale bars, 100um. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.
Figure 2

The C-T network of potential targets. The blue rhombus represent potential targets, purple squares represent drugs and other hexagons represent active ingredients. In addition, nodes' size are proportional to their 'degree'.

A) KXP  
B) CHI  

- 219 (16.8%)  
- 165 (12.6%)  
- 923 (70.6%)
Figure 3

Venn chart and PPI network of potential targets. A. Venn chart conducted using Veooy database. B. PPI network of 81 potential targets performed in Cytoscape 3.7.2. The size of the nodes is proportional to the value of the 'Degree'.

Figure 4

The GO and representative pathways enrichment analysis. A. GO enrichment analysis of 81 potential targets conducted using GraphPad Prism. The green bars represent BP, the purple bars represent CC and the orange bars represent MF. B. The representative pathway enrichment analysis of 81 core targets conducted using GraphPad Prism. The node size represents gene counts and the colors of nodes from red to green illustrate the P value from small to more.
Figure 5

The Target-Pathway (T-P) network. The purple squares represent the potential genes and the red squares represent the signaling pathways. The node size indicates the magnitude of 'Degree'.
Figure 6

The effect of KXP protect against CHI by inhibiting inflammation. A. Gene expression of IL-6. B. Gene expression of IL-113. C. Gene expression of TNF-α. D. Protein expression in serum of IL-6. E. Protein expression in serum of IL-113. F. Protein expression in serum of TNF-α. The results were presented as mean±SD. Hp < 0.05, f< 0.01, vs. the Control group, *p < 0.05, **p < 0.01, vs. the Model group. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.
Figure 7

Effects of ICXY protect against CHI by regulating apoptosis. A. Gene expression of Bax. B. Gene expression of Bcl2. C. Protein bands of Bax and Bcl2. D. Protein expression of Bax. E. Protein expression of Bcl2. Grayscale value of the bands were analyzed using Image J. All results were represented as mean±SD. **p < 0.01, vs. the Control group, *p < 0.05, **p < 0.01, vs. the Model group. ns: no significant. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.

Figure 8

The effect of KXP protect against CHI by regulating PI3K/Akt. A. Protein bands of P13K, Akt and p-AKT. B. Protein expression of PI3K. C. Protein expression of Akt. D. Protein expression of p-Akt. E. Protein expression of p-Akt/Akt. Grayscale value of the bands were analyzed using Image J. The results was presented as mean±SD. < 0.05, vs. the Control group, < 0.05, **p < 0.01, vs. the Model group. ns: no significant. DG: Diammonium Glycyrrhizinate; KXP: Kangxain Pill.