Journal of Ethnopharmacology 257 (2020) 112871

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Network pharmacology approach to elucidate possible action mechanisms of Sinomenii Caulis for treating osteoporosis

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ARTICLE INFO

Keywords:
Sinomenii caulis
Network pharmacology
Osteoporosis

ABSTRACT

Ethnopharmacological relevance: Sinomenii Caulis (SC) is a well-konwn traditional Chinese medicine used for treatment of rheumatoid arthritis (RA), dermatophytosis and paralysis. Patients with RA are usually secondary to osteoporosis, but the potential protective effect of SC on osteoporosis (OP) is seldom reported and its possible action mechanism is little known.

Aim: The purpose of this study was to demonstrate the anti-osteoporosis effects of SC extract and alkaloids in prednisolone (Pre)-induced OP of zebrafish, and then to explore the potential mechanism of SC on system level by network pharmacology.

Methods: Firstly, zebrafish OP model was established to investigate the anti-osteoporosis effect of SC. Secondly, the targets of SC and OP from multiple databases were collected, and Compound-Target-Pathway network based on protein-protein interaction (PPI) was constructed. Moreover, gene enrichment and annotation were performed via the DAVID server. Finally, the reliability of the network pharmacology prediction results in Pre-induced OP of zebrafish was verified by qRT-PCR.

Results: The results indicated that SC extract and alkaloids have remarkable ability to promote bone formation of cranial bones and reduce TRAP contents in Pre-induced OP of zebrafish. 32 OP-related ingredients in SC and 77 OP-related targets were screened from multiple databases, and 15 OP-related pathways were enriched by the KEGG database. Further experimental validation indicated that SC extract and alkaloids could regulate the expression of MAPK14, CASP3, CXCL8, IL-1β, IL6, PTGS2, TNF-α, ESR1, and MMP9 for treatment of OP.

Conclusion: In summary, we conducted an integrative analysis to provide convincing evidence that SC may partially alleviate OP by inhibiting pro-inflammatory cytokines and regulating of RANK/RANKL/OPG system.

1. Introduction

Osteoporosis (OP) is a global health issue closely related to the growth in the aging population, affecting approximately 13% China population (Zeng et al., 2019). It is a systemic skeletal disease characterized by reduced bone mass and impaired microarchitecture. OP is also a common complication in rheumatoid arthritis (RA) patients with higher incidence (Mobini et al., 2012). Bone resorption induced by elevated inflammatory cytokines and bone mass loss induced by glucocorticoid contributed greatly to OP in RA patients (Lun and Xing, 2020). Moreover, oxidative stress, bone immune dysfunction, estrogen deficiency, bone marrow microcirculation disturbance and chronic inflammatory reaction are considered as the causes of OP (Xu et al., 2018a). Many clinical therapies have been used to treat OP, for instance raloxifene, hormone replacement, parathyroid hormone, calcium, testosterone, calcitonin, fluoride, vitamin D and anabolic steroids (De Nijs et al., 2006; Jacobs et al., 2007). However, most conventional anti-osteoporotic drugs have disadvantages such as poor efficacy and serious adverse effects.

Traditional Chinese medicines show great potential in the prevention and treatment of OP because of their rich resources, low price, good curative effect and low side effect. Sinomenii Caulis (SC) is a well-konwn Chinese medicine used for treatment of rheumatoid arthritis, dermatophytosis and paralysis (Jiang et al., 2019). Phytochemical studies demonstrated that SC contains abundant alkaloids with various skeletons, as well as lignans and cyanoglucoside. Sinomenine is the main bioactive component of SC, which was reported to possess a wide range of pharmacological actions, such as anti-RA, immunosuppressive,

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https://doi.org/10.1016/j.jep.2020.112871
Received 5 March 2020; Received in revised form 9 April 2020; Accepted 9 April 2020
Available online 20 April 2020
0378-8741/

Abbreviations

ALP	Alkaline phosphatase
AMB	The area of mineralized bones
CASP3	Caspase-3
COD	Cumulative optical density
Ed	Etidronate disodium
Deh	Dehydrodiscretine
ELISA	Enzyme-linked immunosorbent assay
ESR1	Estrogen receptor 1
Ext	Ethanol extract of SC
IL6	Interleukin-6
IL8/CXCL8	Interleukin-8
IL1β	Interleukin-1 beta
Mag	Magnoflorine
MAPK14	Mitogen-activated protein kinase 14
MMP9	Matrix metalloproteinase-9
NOS3	Nitric oxide synthase 3
OP	Osteoporosis
OPG	Osteoprotegerin
RANK	Nuclear factor-κB receptor activating factor
RANKL	Nuclear factor-κB receptor activating factor ligand
PPI	Protein-protein interaction
Pre	Prednisolone
PTGS2	Prostaglandin G/H synthase 2
SC	Sinomenii Caulis
Sin	Sinomenine
Ste	Stepharanine
Tal	Total alkaloid extract
TNF-α	Tumor necrosis factor alpha
TRAP	Tartrate resistant acid phosphatase

anti-angiogenic, analgesic activities, suppression of osteoclast formation and bone loss (Cheng et al., 2009; Kok et al., 2005; Li et al., 2013; Wang and Li, 2011). Lee et al. reported that the isoquinoline alkaloids in SC could inhibit osteoclast differentiation in *vitro* (Lee et al., 2016). However, researches on the anti-osteoporotic activities of SC in *vivo* are lacking, and its possible pharmacological mechanism is not clear.

Generally, the complex components in traditional Chinese medicines exert their pharmacological effect through a multi-target and multi-pathway, which can hardly be elucidated by traditional methods (Zhang et al., 2015). Network pharmacology is a integrity, synergy and dynamics analysis method based on disease, gene, protein target and drug interaction network (Hopkins, 2008). It could be utilized as a valid tool to elucidate the pharmacological mechanism of SC at a holistic level (Li et al., 2012; Zhao et al., 2009).

Since the structure and genetics of zebrafish bone are nearly identical with human, the zebrafish mode have acquired increasing attention in the study of bone deformations and dysplasias (Yin et al., 2018). Herein, we firstly evaluated the effects of SC extract and alkaloids in prednisolone (Pre)-induced OP of zebrafish. Then, the potential anti-OP mechanism of SC was further studied by network pharmacology strategy. Finally, the crucial targets from pathway enrichment analysis were experimentally validated to elucidate the potential mechanism of SC against OP.

2. Materials and methods

2.1. Chemicals and reagents

SC is the dried stems and rhizomes of *Sinomenium acutum* (Thunb.) Rehd. et Wils.or *Sinomenium acutum* (Thnnh.) Rehd. et Wils. var. cinereum Rehd.et Wils. The SC sample was purchased from Hubei Province (China), and was identified by Professor E-Hu Liu (Pharmacognosy Department, China Pharmaceutical University). And the HPLC chemical fingerprint of the SC sample was shown in Fig. S1 by our previous method (Jiang et al., 2019). The voucher specimen (HB-1, S11) has been deposited in the State Key Laboratory of Natural Medicines, China Pharmaceutical University.

Prednisolone (Pre) and etidronate disodium (Ed) were obtained from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Sinomenine (Sin, CAS: 115-53-7), magnoflorine (Mag, CAS: 2141-09-5), stepharanine (Ste, CAS: 17369-30-1) and dehydrodiscretine (Deh, CAS: 68947-61-5) were isolated and purified from SC in our laboratory. The zebrafish tartrate resistant acid phosphatase (TRAP) enzyme-linked immunosorbent assay (ELISA) Kit and zebrafish alkaline phosphatase (ALP) ELISA Kit were purchased from Shanghai Mlbio Biotechnology Co., Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Shanghai Titan Scientific Co., Ltd. (Shanghai, China). Alizarin red was purchased from Tianjin Chemical Reagent Research Institute (Tianjin, China).

2.2. Sample preparation

The dried and chopped SC was refluxed twice with 95% ethanol and filtered. The filtrate was evaporated under decompression to obtain the ethanol extract of SC (Ext). Total alkaloid extract (Tal) was prepared using the methods that reported in our previously published article (Wang et al., 2019).

2.3. Animals

The wild type AB strain zebrafish was supplied by Nanjing YSY biotechnology Co., Ltd. (Nanjing, China) and maintained according to standard conditions (14:10 h light/dark cycle at 28 °C and fed twice daily with brine shrimp) described in the Zebrafish Book (Chen et al., 2018). Next, normally fertilized embryos were collected from nature crosses of 3 ~ 4 pairs of adult zebrafish and maintained in the zebrafish embryo culture solution for further analysis. Animal experiments were conducted in accordance with the Guidelines for Animal Experiments of China Pharmaceutical University and approved by the Animal Ethics Committee of this university.

2.4. Experimental procedures

The dose of 25 μM Pre was used in subsequent experiment on bone formation in zebrafish larvae according to a previous report (Liu et al., 2018). The 3 dpf (days past fertilization) zebrafish larvae were raised in 24-well plate (n = 12 larvae/2 wells/group) and randomly divided into different groups as follows: 0.2% DMSO (control group), 25 μM Pre (model group), 25 μM Pre + 120 μM Ed (positive group), 25 μM Pre + Ext (200, 400, 800, 1600, 3200 μg/mL), 25 μM Pre + Tal (200, 400, 800, 1600, 3200 μg/mL), 25 μM Pre + Sin (25, 50, 100, 200, 400 μM), 25 μM Pre + Ste (25, 50, 100, 200, 400 μM), 25 μM Pre + Mag (25, 50, 100, 200, 400 μM), 25 μM Pre + Deh (25, 50, 100, 200, 400 μM) from 3 dpf to 9 dpf. Replace one half of the liquid in every plate/day (1.5 mL). At the end of the experiment, the zebrafish were carefully harvested for following examination.

2.5. Assessment of anti-osteoporosis activity

2.5.1. Skeletal staining

Bone mineralization matrix deposition is an important index of bone formation, which is evaluated by alizarin red staining. All the zebrafish were sacrificed by 0.02% tricaine, at the 9 dpf. After removal of tricaine solution, zebrafish larvae were fixed in 4% paraformaldehyde for 2 h

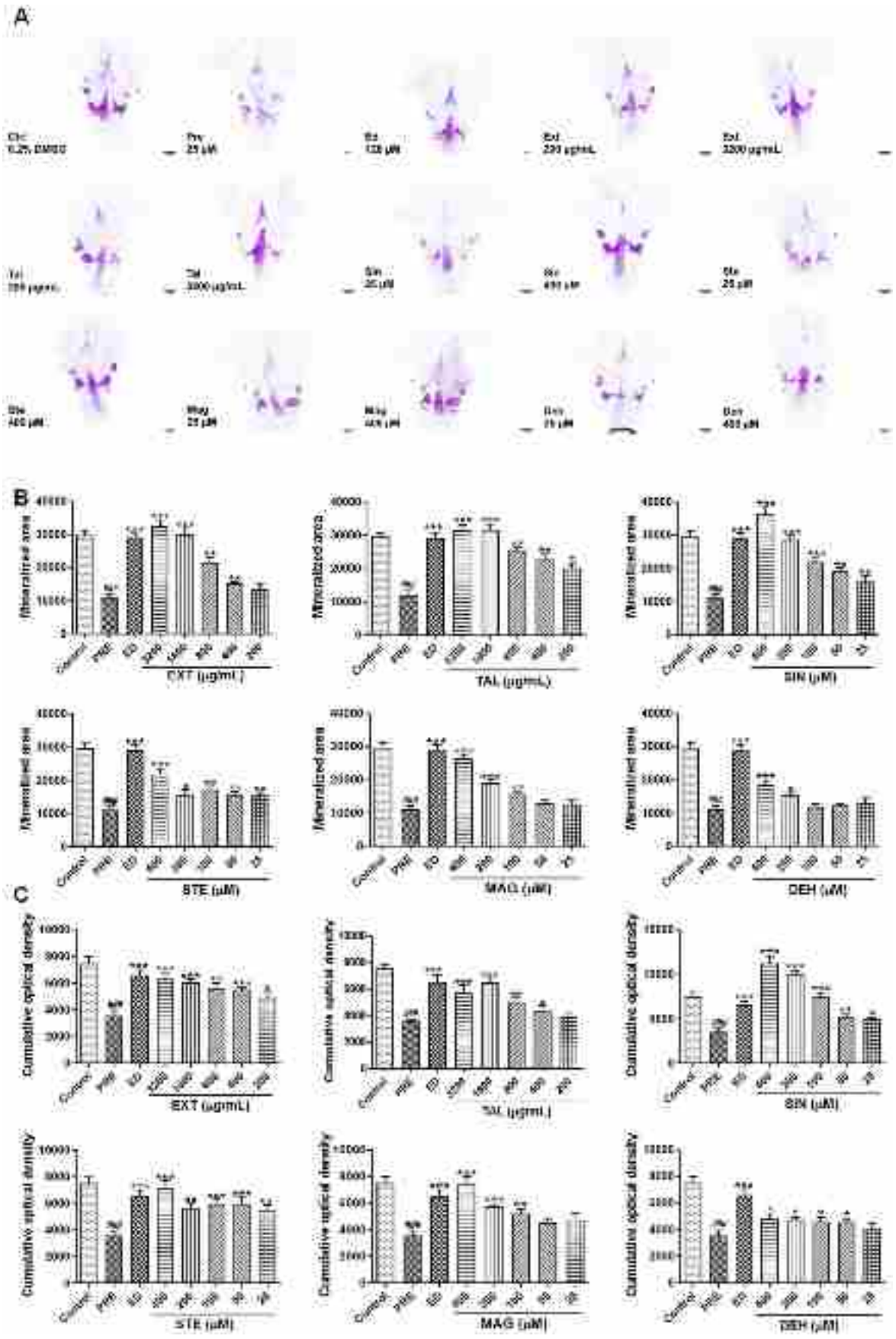

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Fig. 1. The effects of SC (Ext, Tal, Sin, Ste, Mag and Deh) on bone mineralization of wild-type AB strains zebrafish larvae skull. Images of (A) the dorsal aspect head bone stained with alizarin red in wild type AB strains zebrafish larvae at 9-days postfertilisation (dpf) with or without exposure to SC extract and alkaloids (Ext and Tal: 200 μg/mL, 3200 μg/mL; alkaloids: 25 μM, 400 μM); (B) the effect of SC on bone mineralization area in 9-dpf zebrafish; and (C) the effect of SC on bone mineralization cumulative optical density in 9-dpf zebrafish. Data are given as mean ± standard deviation (n = 9). $^{###}$Compared with Control, $p < 0.001$. *Compared with Pre, $p < 0.05$. **Compared with Pre, $p < 0.01$. ***Compared with Pre, $p < 0.001$. The mineralized tissue is stained in red.

and bleached to transparency with fresh prepared decolorizer (3% H_2O_2, 0.5% KOH) for 1 h. Next, the zebrafish larvae were dehydrated in 50% ethanol for 10 min and stained with 0.1% Alizarin red stain (ARS, 0.5% KOH) to stain formed bone overnight. In addition, the zebrafish were transparent with 0.5% KOH and glycerin with a gradient ration of 3:1 to remove excess stain for 6 h, and then replaced washes with 0.5% KOH and glycerin (1:1) for 12 h, and subsequently changed to 0.5% KOH and glycerin (1:3) for 12 h. Finally, all samples were decolored with glycerin and the stained zebrafish heads were placed on a slide, and images of the dorsal aspect head bone were photographed using a fluorescence stereomicroscope (OLYMPUS, SZX16, Japan). The mineralized bone area (AMB) and cumulative optical density (COD) were calculated by Image-ProPlus 6.0 image analysis software to reflect bone mineralization and bone mineral density.

2.5.2. TRAP and ALP activity

The TRAP and ALP activity were detected by using zebrafish TRAP ELISA Kit (mlbio) and zebrafish ALP ELISA Kit (mlbio).

2.6. Network pharmacology study

2.6.1. Screening of active ingredients of SC

The components of SC were acquired from Traditional Chinese Medicine Systems Pharmacology Database (TCMSP, http://lsp.nwsuaf.edu.cn/tcmsp.php) and existing literature. Afterward, two key ADME indexes including oral bioavailability (OB ≥ 30%) and drug similarity (DL ≥ 0.18) were employed to screen the candidate active compounds in SC (Pang et al., 2018). Moreover, several active constituents with relative high contents or excellent bioactivities, which did not satisfy these two criteria, were also manually supplemented as the candidate compounds for further analysis (Jiang et al., 2019).

2.6.2. Compound-related targets prediction

In this study, a comprehensive compound targeting method was established to find the targets of SC. First, the potential targets were obtained from STITCH (score ≥ 0.5, http://stitch.embl.de/), BATMAN-TCM (Score cutoff ≥ 20, http://bionet.ncpsb.org/batman-tcm/) and Swiss Target Prediction (p > 0.5, http://www. swisstargetprediction.ch/). After redundancy was deleted, the related targets of candidate compounds were imported into Google Scholar and Pubmed Database, and relevant literatures were searched for verification.

2.6.3. OP-related targets prediction

Known OP-related genes were obtained from five existing resources: GeneCards (http://www.genecards.org); Comparative Toxicogenomics Database (CTD, Inference Score ≥ 20, http://ctdbase.org); OMIM database (Online Mendelian Inheritance in Man; http://www.omim. org/); DisGeNet database (http://www.disgenet.org/); and TTD database (http://database.idrb.cqu.edu.cn/TTD/). After redundancy was deleted, the putative targets were validated one by one on the Google Scholar, Pubmed Database.

2.6.4. KEGG pathway enrichment

DAVID (version 6.8, https://david.ncifcrf.gov/) was performed to conduct KEGG pathway enrichment analysis. Those pathway terms with a p-value of ≤0.05 and Benjamini value of ≤0.5 were regarded as significant and interesting.

2.6.5. Constructing the network for SC intervention OP

Based on above identification results of compounds and target, combining with Protein–Protein Interaction (PPI) data in STRING (https://string-db.org/) and KEGG pathway enrichment analysis date, the Compound-Target (C-T), Target-Pathway (T-P) and Compound-Target-Pathway (C-T-P) network models were constructed by using Cytoscape 3.7.1 software (Institute for Systems Biology, Seattle, WA, USA). Subsequently, the parameters "Degree centrality (DC)" and "Betweenness centrality (BC)", etc.) of the network topology, which were used to demonstrate the topological importance of nodes, were calculated by the Network Analyzer plugin in Cytoscape.

2.7. RNA isolation and quantitative real-time PCR

The total RNA of zebrafish larvae (n = 30 larvae/group) was extracted with Trizol reagent (Vazyme, Nanjing, China) according to the instructions of the manufacturer. Then, the cDNAs were transcribed using high capacity cDNA reverse transcription kit (Hiscript II reverse transcriptase, Vazyme, Nanjing, China). qRT-PCR was performed in Roche Light Cycler 96 QPCR System (Roche, Switzerland) using the SYBR-green system (Vazyme, Nanjing, China). Gene-specific primers used for real-time reactions were obtained from Sangon Biotech and shown in Table S1.

2.8. Statistical analysis

GraphPad Prism Version 8.00 was used for statistical analysis. All quantitative data were presented as the mean ± SD. Results were analyzed by one-way ANOVA for multiple group comparisons and Student's t-test for two-group comparisons.

3. Results

3.1. SC exhibits anti-osteoporosis effect in zebrafish

Considering the anti-inflammatory components isolated from SC in our previous study (Wang et al., 2019), the Ext, Tal and four major alkaloids including sinomenine, magnoflorine, stepharanine and dehydrodiscretine were selected to investigate the effect of SC on the osteogenesis of zebrafish larvae. The model of Pre-induced OP zebrafish were exposed to either 3200, 1600, 800, 400 and 200 μg/mL Ext or Tal, 400, 200, 100, 50 and 25 μM Sin, Ste, Mag or Deh from 3 dpf to 9 dpf, respectively. As shown in Fig. 1, comparing with the normal group, the AMB and COD in Pre-induced model showed a significant decrease ($P < 0.05$). Comparing with the model group, 120 μM etidronate disodium could reverse the decrease of AMB and COD ($P < 0.05$). The SC Ext (400 ~ 3200 μg/mL), Tal (200 ~ 3200 μg/mL), Sin (25 ~ 400 μM), Ste (25 ~ 400 μM), Mag (100 ~ 400 μM), and Deh (200 ~ 400 μM) suppressed the inhibition of prednisolone on bone formation in zebrafish larvae ($P < 0.05$). It was found that Tal exhibited stronger activity to attenuate the prednisolone-induced inhibition of bone mineralization than Ext, and Sin showed the strongest activity, followed by Ste, Mag and Deh. In summary, the results suggested that SC has the capacity to promote cranial bone formation in zebrafish larvae.

ALP and TRAP are well-recognized biochemical markers of osteoblast and osteoclastic differentiation. In order to explore the relationship between the decrease of mineral content and the activity of osteoclast and osteoblast, we evaluated the effects of two typical markers of bone resorption in zebrafish larvae. As shown in Fig. 2, pretreatment

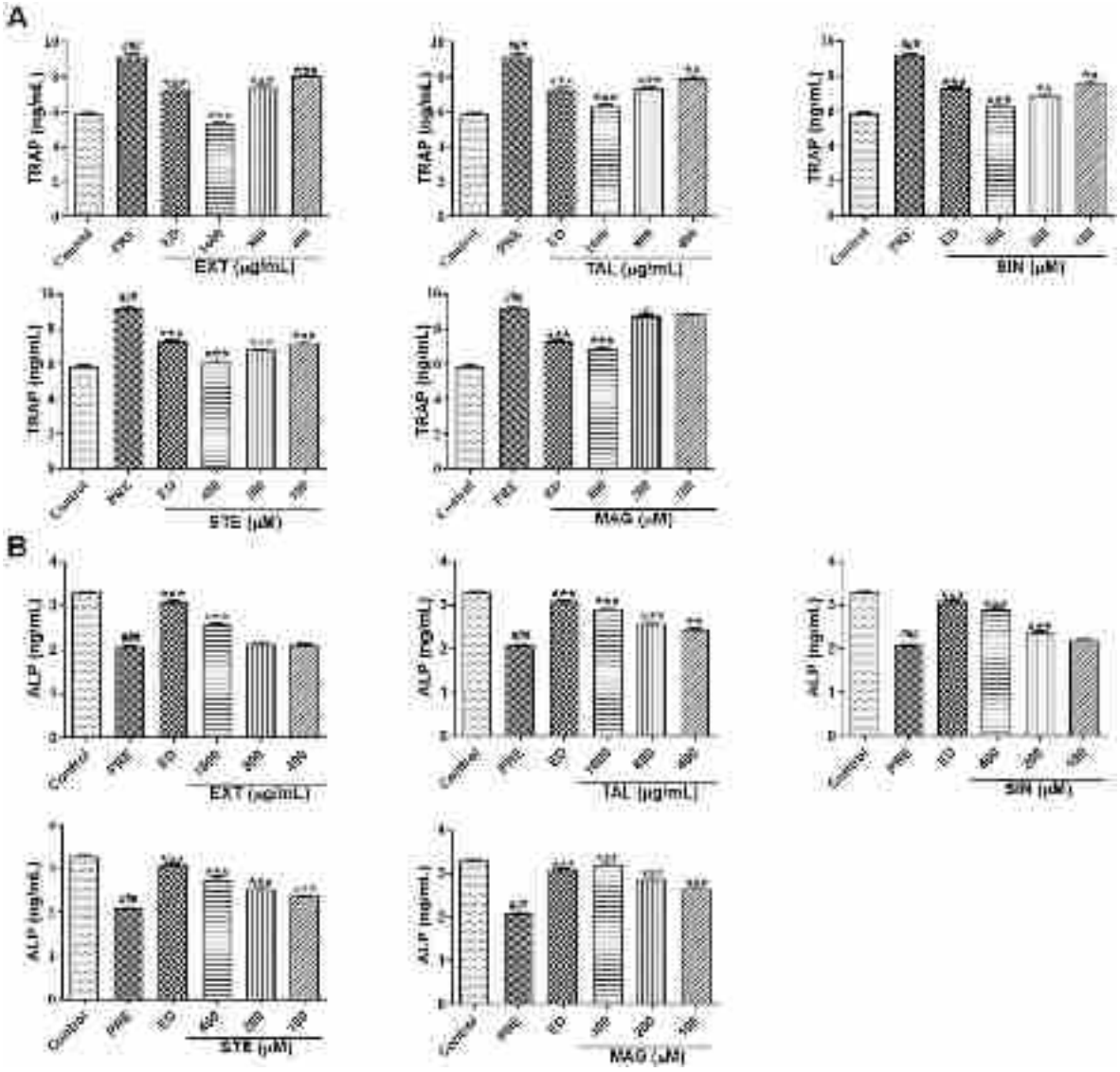

Fig. 2. The effect of SC (Ext, Tal, Sin, Ste, and Mag) on TRAP (A) and ALP (B) activity in 9-dpf zebrafish. Data are given as mean ± standard deviation ($n = 6$). $^{###}$Compared with Control, $p < 0.001$. *Compared with Pre, $p < 0.05$. **Compared with Pre, $p < 0.01$. ***Compared with Pre, $p < 0.001$.

with 25 μM Pre significantly increased TRAP activity and decreased ALP activity in zebrafish compared with the control group ($P < 0.05$), meanwhile the positive control etidronate disodium reversed this effect. Moreover, zebrafish treated with Ext, Tal, Sin, Ste, Mag, and Deh showed a tread toward reduction of the TRAP activity and increase of the ALP activity ($P < 0.05$). These results indicated that SC could ameliorate Pre-induced inhibition of osteoblastic differentiation and promotion of osteoclastic differentiation.

3.2. Network pharmacology analysis

3.2.1. Active ingredients of SC

A total of 93 potential compounds were initially collected from TCMSP, TCMD and existing literature. Combining with the ADME profiles and literature confirmation, 32 candidate ingredients were finally selected for further analysis (Table 1). The manually supplemented compounds were validated with reported pharmacological research. For example, morphine alkaloids in SC, such as sinomenine and the analogues of sinomenine (isosinomenine, 2,2-disinomenine, and Sinomenine *N*-oxide), have been reported that have anti-angiogenic, anti-inflammatory and anti-rheumatic effects (Kok et al., 2005). Moreover, higenamine plays a key role in the treatment of inflammatory diseases.(Zhang et al., 2017a).

3.2.2. Target prediction

Generally, the effectiveness of the SC to prevent OP depends on the synergistic effects between multiple compounds and their targets. Thus, it is necessary to explore the targets based on predicting ingredients. A comprehensive predictive method combining STITCH, BATMAN-TCM and Swiss Target Prediction was subsequently intended to predict 101 putative targets, which interacted with 32 candidate compounds. The CTD combining GeneCards, OMIM, DisGeNet, and TTD database was performed to predict 4,774 targets associated with OP. Then, 87 targets were screened by finding the overlapping targets from the compound-related targets and OP-related targets. A higher score in CTD and GeneCards databases indicated a higher correlation with OP. The arithmetic average of the above databases was selected as integration scores to screen the candidate targets. As the result in Table S2, the top 77 targets with Inference Score ≥20 were used to further research.

3.2.3. Network construction and node screening

Using PPI networks can understand the function of diverse targets in complex diseases, such as OP. Therefore, we constructed a network consisting of candidate compounds and targets (C-T, 109 nodes and

Table 1
The candidate compounds of SC.

NO.	Molecule ID	Molecule name	OB (%)	DL
M1	MOL000625	Sinomenine	46.09	0.53
M2	MOL012905	Disinomenine	17.97	0.1
M3	–	Isosinomenine	–	–
M4	MOL001779	Salutaridine	49.11	0.46
M5	–	Sinomenine N-oxide	–	–
M6	–	Cephamonine	–	–
M7	–	2,2-disinomenine	–	–
M8	MOL004347	Laurifoline	22.92	0.55
M9	MOL000794	Menisperine	26.17	0.59
M10	–	Corydine methyl ether	–	–
M11	MOL012910	Bianfugecine	36.08	0.42
M12	MOL000782	Menisporphine	24.33	0.52
M13	MOL012918	dauriporphinoline	33.54	0.57
M14	MOL012912	Bianfugenine	27.39	0.61
M15	MOL000627	Stepholidine	33.11	0.54
M16	MOL000790	Isocorypalmine	35.77	0.59
M17	MOL009149	Cheilanthifoline	46.51	0.72
M18	MOL006443	Stepharanine	77.79	0.54
M19	MOL001455	(S)-Canadine	53.83	0.77
M20	MOL004071	Hyndarin	73.94	0.64
M21	MOL004205	Dehydrocorydalmine	43.9	0.59
M22	MOL001457	Columbamine	26.94	0.59
M23	MOL000785	Palmatine	64.6	0.65
M24	MOL002904	Berlambine	36.68	0.82
M25	–	Sinactine	–	–
M26	–	Salsolidine	–	–
M27	–	Higenamine	–	–
M28	MOL001522	Coclaurine	42.35	0.24
M29	MOL000764	Magnoflorine	26.69	0.55
M30	–	Magnocurarine	–	–
M31	MOL000621	16-epi-Isositsirikine	49.52	0.59
M32	MOL012904	Dauricumine	52.86	0.44

"–" NO useful data was searched.

1164 edges) using the Cytoscape combined with PPI data (Fig. S2). As shown in Fig. 3, Sin (M1), Ste (M18), Corydine methylether (M10) and Mag (M29), were the main active ingredients of SC with high-node degree, and the anti-OP effect of Sin, Ste, Mag have been verified in pharmacological study. Moreover, the genes CASP3, IL6, TNF-α, PTGS2, MAPK14, IL1β, IL8/CXCL8, NOS3, MMP9, and ESR1 with high-node degree were considered as the key targets regulated by SC.

3.2.4. KEGG pathway enrichment analysis

To further investigate the anti-OP mechanisms of SC, the top 30 candidate targets with high-node degree (C–P network) were selected for KEGG pathway enrichment analysis by the DAVID Bioinformatics Resources 6.8 software. Due to diseases were caused by basic biological dysfunctions, we removed the KEGG pathway section of human diseases (Xu et al., 2018b). After filtering by a parameter p-value cutoff of ≤ 0.05, 15 KEGG pathway terms were obtained and a bubble diagram was constructed in Fig. 4. As shown in Table 2, CASP3, IL6, TNF, PTGS2, MAPK14, IL1β, TLR4, NOS3, ACHE and ESR1 were the most frequently occurring protein targets. According to the pathogenesis of OP, these KEGG pathway terms could be divided into 5 modules including inflammatory response (TNF signaling pathway), immune response (T cell receptor signaling pathway), cell apoptosis (PI3K-Akt signaling pathway), bone metabolism (Osteoclast differentiation), and endocrine system (Estrogen signaling pathway). Based on these results, SC may attenuate OP partially by regulating inflammatory response, immune response, bone metabolism, and numerous cell apoptosis KEGG pathways during OP progression.

3.2.5. An integrated network model analysis

According to the screening results of the candidate compounds, top 30 related targets, and KEGG pathways, an integrated C-T-P network (Fig. 5) was constructed by combining with the PPI network. In the C-T-P network, nodes with a shorter average shortest path length and higher betweenness centrality in C-T-P network were considered as vital ones. As shown in the results, PTGS2, NOS3, MAPK14, IL6, and TNF-α were the top 5 nodes, consistent with the result of C–P network. Moreover, Sin, Ste, Mag and Corydine methylether made more contribution to attenuate OP by regulating the top 10 key targets. It's worth noting that aporphines alkaloids have high-node degree than other type alkaloids. Later, Ext, Sin and Ste was selected to verify whether SC exerted its effects by regulating the key targets and pathways, the expression of OP-related top 10 targets in zebrafish larvae was detected using qRT-PCR assay.

3.3. Results of biological verification

The network pharmacology results offered certain potential key targets and pathways of SC to ameliorate OP. To verify the reliability of the network pharmacology prediction results, the mRNA levels of these key genes in larval zebrafish, including CASP3, IL6, TNF-α, PTGS2, MAPK14, IL1β, CXCL8, NOS3, MMP9 and ESR1, were assessed by qRT-PCR. As shown in Fig. 6, comparing to the corresponding control group, the mRNA expression of CASP3, IL6, TNF-α, PTGS2, MAPK14, IL1β, CXCL8, NOS3, MMP9 and ESR1 were significantly increased in zebrafish larvae after being treated with 25 μM Pre ($P < 0.05$). Conversely, 800 μg/mL EXT, 200 μM SIN, and 200 μM STE reduced the Pre-induced up-regulation of osteoclast-specific genes. These results further demonstrated that SC, Sin and Ste could rescue the Pre-induced inhibition of osteogenesis by down-regulating the expression of osteoclast-specific genes.

4. Discussion

It has been well acknowledged that traditional Chinese medicines play an important role in health maintenance in China and abroad. However, the mechanisms of action of Chinese medicines are often

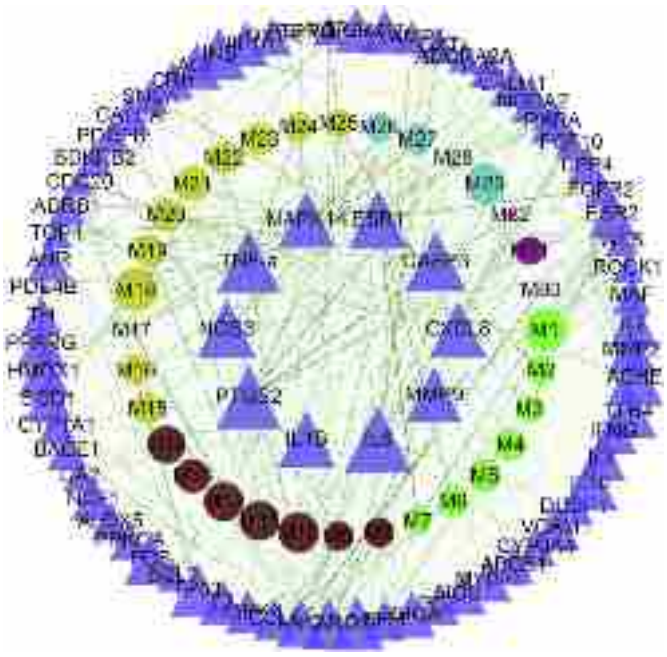

Fig. 3. The Compound-Target network combine with putative targets PPI data. Circle nodes in different colors represent candidate compounds from SC that are grouped together by structural category, and blue triangle nodes represents the candidate targets that were directly associated with candidate compounds. The size of nodes was proportional with the degree. Green circle represents morphinans alkaloids, brown circle represents aporphines alkaloids, yellow circle represents protoberberines alkaloids alkaloids, wathet blue circle benzylisoquinolines alkaloids, and violet circle represents other alkaloids.

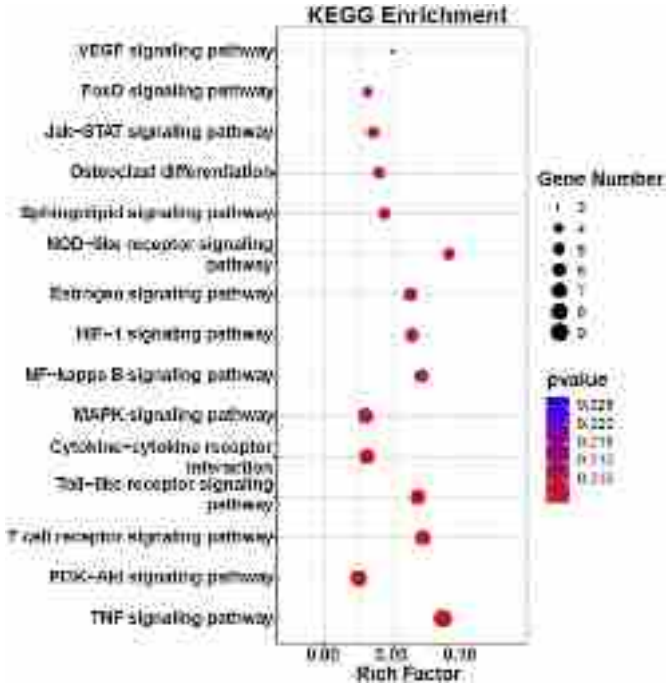

Fig. 4. KEGG pathway enrichment bubble diagram for the top 30 candidate targets ($P \leq 0.05$).

elusive due to the components contained. It is of great significance to integrate network pharmacology, which is based on big data bioinformatics, to uncover the molecular mechanism of Chinese medicines.

OP is a common form of secondary osteoporosis and has led to an increased risk of fracture in many patients. Zebrafish is considered to be a powerful model for the study of bone disease because of its bone structure, bone metabolism and signal pathway, which are highly similar to those of human beings (Zhao et al., 2020). *Barrett, R.* reported that the zebrafish larvae exhibit a significant delay in the early process of mineralization (Barrett et al., 2006) after treating with Pre. Both osteoblasts and osteoclasts have been identified in zebrafish and found to be functionally related to those in humans. Therefore, the Pre-induced zebrafish OP model has acquired an increasing attention.

Our results demonstrated that SC extract and some alkaloids including Sin, Ste, Mag and Deh exhibited potent activity to promote bone formation of cranial bones in zebrafish larvae. It was proposed that ALP promoted bone mineralization and TRAP accelerated bone resorption (Pasqualetti et al., 2015). SC extract and alkaloids had the ability to increase ALP activity and reduce TRAP activity by suppressing the expression of osteoclastogenic genes and exciting the expression of osteoblastogenic genes in zebrafish, which indicated a promising therapeutic effect of SC on OP. However, the molecular mechanism of SC alkaloids in the treatment of OP is not clear. In this study, we employed the Pre-induced OP model of zebrafish larvae to verify the effect of SC. At the same time, the compounds, targets and KEGG pathways of SC on OP were reviewed by the method of network pharmacology. Lastly, the selected target genes were verified based on quantification of the expression of top 10 OP-related genes.

We collected 93 native SC compounds from TCMSP, TCMD and existing literature, and 32 compounds were selected by ADME and manually supplemented. Further calculations using STITCH, BATMAN-TCM, and Swiss Target Prediction identified 101 target proteins, 77 of which were obtained by screening criteria. From the CTD, GeneCards, OMIM, DisGeNet, and TTD databases, we collected 4774 OP-associated gene entries. Based on the integration score, the top 30 putative target genes were selected to further explore the KEGG pathway. 15 KEGG pathway terms were returned and classified, and 3 networks were constructed and integrated. Having identified the candidate targets based on network analysis, we further verified the reliability of the network pharmacology prediction results. Our data highlighted that SC inhibited OP by regulating inflammatory response, immune response, endocrine system, cell apoptosis and bone metabolism related pathways through MAPK14, PTGS2, IL-1β, IL6, MMP9, and ESR1.

The relationship between OP and inflammation response has been studied for decades. Previous studies have showed that many pro-inflammatory cytokines including IL-6 (Steeve et al., 2004), TNF-α (Redlich et al., 2002), IL-8 (Gur et al., 2002), IL-1β (Sipos et al., 2010) and IL-17 (Kotake et al., 1999), which active in inflammation and immunity, play a role in physiologic and pathologic resorption of bone. Thus, the inhibition of inflammation is critical in treating OP. IL-1β, IL-6, CXCL8, and TNF-α belonged to the pro-inflammatory cytokines and were down-regulated by Ext, Sin and Ste in Pre-induced OP of zebrefish (Fig. 6). Pro-inflammatory cytokines are not only the most effective locally produced bone resorption stimulators, but also recognized as bone formation inhibitors. TNF-α can induce bone marrow stromal cells to produce cytokines such as RANKL, M-CSF and IL-6, and promote the formation and activation of osteoclasts. These cytokines increase the recruitment of osteoclasts by acting on hematopoietic stem cells, resulting in bone injury of OP. Therefore, blocking the effect of pro-inflammatory cytokines could effectively reduce the inflammatory response and bone destruction of OP.

PTGS2 also plays a key role in regulating the osteogenesis of mesenchymal stem cells differentiation into osteoblast lineage and osteoclast maturation through the COX2-PGE2 pathway (Lu et al., 2017). In biological verification data by qRT-PCR, PTGS2 was up-regulated in Pre-induced OP of zebrafish. Moreover, the regulating effect of Ext, Sin

Table 2
The classification results of KEGG pathway enrichment analysis.

Category	KEGG Pathway Terms	Gene	p-Value	Benjamini
Inflammatory response	TNF signaling pathway	VCAM1, FOS, CASP3, IL6, TNF, PTGS2, MAPK14, MMP9, IL1β	8.6E-09	3.2E-07
Immune response	T cell receptor signaling pathway	IL4, FOS, TNF, MAPK14, RHOA, IL10, IL2	2.9E-06	3.1E-05
Immune response	Toll-like receptor signaling pathway	FOS, IL6, TNF, MAPK14, CXCL8, IL1β, TLR4	4.1E-06	4.1E-05
Inflammatory response	NF-kappa B signaling pathway	VCAM1, TNF, PTGS2, CXCL8, IL1β, TLR4	2.7E-05	2.2E-04
Inflammatory response	HIF-1 signaling pathway	IL6, INS, HMOX1, TLR4, NOS3, NOS2	4.3E-05	3.2E-04
Endocrine system	Estrogen signaling pathway	FOS, MMP9, ESR1, NOS3, ESR2, MMP2	5.0E-05	3.5E-04
Inflammatory response	NOD-like receptor signaling pathway	IL6, TNF, MAPK14, CXCL8, IL1β	8.0E-05	5.2E-04
Inflammatory response	Cytokine-cytokine receptor interaction	IL4, IL6, TNF, CXCL8, IL1β, IL10, IL2	4.4E-04	2.2E-03
Cell apoptosis	PI3K-Akt signaling pathway	IL4, IL6, INS, TP53, TLR4, NOS3, MYC, IL2	4.5E-04	2.1E-03
Cell apoptosis	MAPK signaling pathway	FOS, CASP3, TNF, MAPK14, TP53, IL1β, MYC	5.4E-04	2.4E-03
Endocrine system	Sphingolipid signaling pathway	TNF, MAPK14, TP53, RHOA, NOS3	1.5E-03	5.3E-03
Bone metabolism	Osteoclast differentiation	FOS, TNF, MAPK14, PPARG, IL1β	2.1E-03	7.1E-03
Cell apoptosis	Jak-STAT signaling pathway	IL4, IL6, MYC, IL10, IL2	3.0E-03	1.0E-02
Cell apoptosis	FoxO signaling pathway	IL6, INS, MAPK14, IL10	1.8E-02	5.1E-02
Inflammatory response	VEGF signaling pathway	PTGS2, MAPK14, NOS3	2.7E-02	7.1E-02

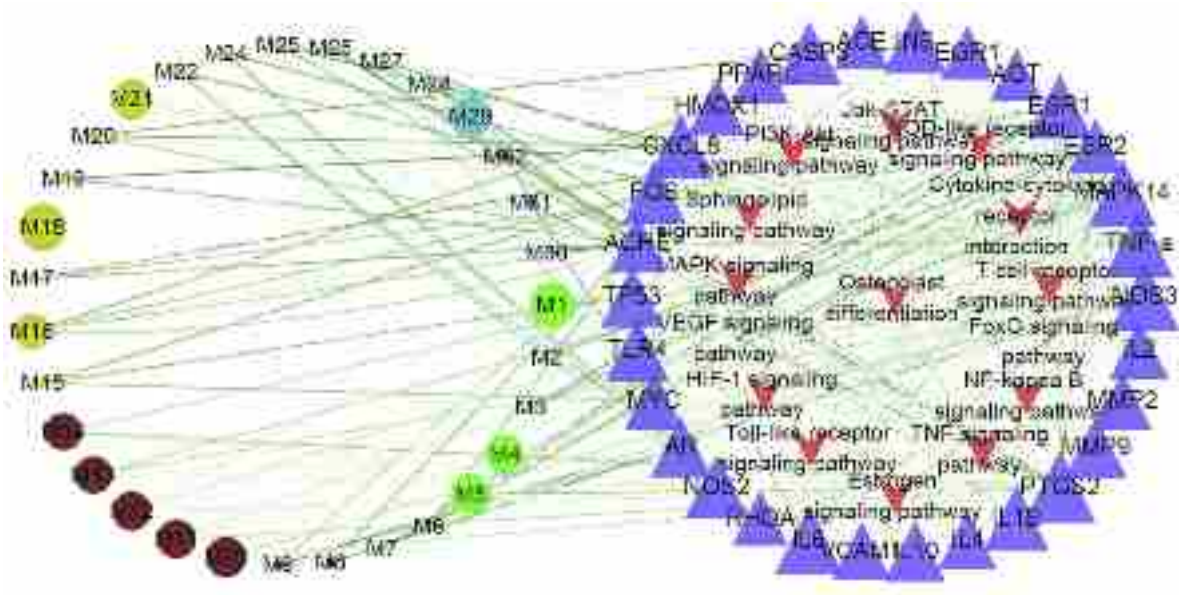

Fig. 5. The Compound–Target–Pathway (C-T-P) network about compounds, putative targets and 15 KEGG pathways. Circle nodes in different colors represent different groups of compounds, blue triangle nodes represents the candidate targets, and orange "V" nodes represent the KEGG pathways. The size of nodes was proportional with the degree and larger sizes represent greater associations with OP.

and Ste was validated *in vivo* (Fig. 6). Most of the SC compounds obtained in STITCH, BATMAN-TCM, and Swiss Target Prediction could interact with PTGS2, particularly Sin and Ste have higher affinity with PTGS2 target. Sin could inhibit the formation of mesenchymal stem cells (MSCs) *in vitro* and suppress the expression of Prostaglandin E2 (PGE2), a key regulator of osteoclast fusion (Zhou et al., 2017). Therefore, inhibiting the expression of PTGS2 can also reduce the inflammatory response and bone destruction of OP.

NOS3 was downregulated with treatment of SC, which has been shown to decrease osteoclastogenesis and bone resorption through reducing the ratio of RANKL/osteoprotegerin (OPG) (Cho et al., 2008). The network pharmacology analysis suggest that most SC compounds could affect bone metabolism and have a strong affinity with NOS3. Consistent with this prediction, our studies showed that Ext, Sin and Ste could down-regulate the expression of NOS3 in Pre-induced OP of zebrafish (Fig. 6). Therefore, SC could decrease the activity of osteoclasts by inhibiting the expression of NOS3, and achieve the pharmacological effect of interfering OP. In the pre-induced zebrafish OP model, the activation of osteoclasts was significantly related to the increased expression of MMP9, and inhibition of MMP9 could significantly restore bone volume (Kim et al., 2017). It has been previously determined that the function of MMP9 could be expressed as osteoclast markers (Zhang et al., 2017b). Our data suggested that MMP9 could be activated by many SC compounds, such as Sin, Ste and Mag which act via different signaling pathways. In the research of Li X. J. study (Li et al., 2013), Sin could inhibit osteoclast formation and bone resorption induced by NF-κB ligand (RANKL), and could dose-dependently inhibit osteoclast specific marker genes induced by RANKL, including MMP-9, TRAP. To sum up, SC could significantly decrease osteoclast differentiation and bone resorption by inhibiting pro-inflammatory cytokines and regulating RANK/RANKL/OPG system.

Excess of estrogen could lead to pathological conditions such as infertility, cancer, and osteoporosis (Xin et al., 2019). Estrogen receptor 1 (ESR1) is a hormone regulator of bone metabolism (Masi et al., 2014). Moreover, ESR1 and mitogen activated protein kinase 3 (MAPK3) network have been proposed as a cause of increased osteoclast genesis and decreased osteoblast genesis (Xiao et al., 2010). ESR1 and MAPK14 belonged to the ERs and were down-regulated by Ext, Sin and Ste in Pre-induced OP of zebrefish (Fig. 6). According to the results of C-T-P network, Sin and Ste had higher affinity for ESR1 and MAPK14, which might play an important role in the anti-OP effects of SC. To sum up, SC and the alkaloids of SC could alleviate OP by regulating inflammatory reaction and estrogen secretion to inhibit bone resorption.

5. Conclusions

In the present study, the network pharmacology approach was adopted for the first time to explore the underlying mechanism of SC on OP. Studies on the Pre-induced OP of zebrafish model showed that SC extract and alkaloids had significant anti-osteoporosis activities. By network pharmacology analysis, the results demonstrated that the anti-OP mechanism of SC might be through modulation of the TNF signaling pathway, PI3K-Akt signaling pathway, T cell receptor signaling pathway, Osteoclast differentiation, etc. The anti-OP effect of SC extract and alkaloids might be due to inhibiting of pro-inflammatory cytokines and regulating of RANK/RANKL/OPG system by down-regulation of MAPK14, CASP3, CXCL8, IL-1β, IL6, PTGS2, TNF-α, ESR1, and MMP9. Our study demonstrated the reliability of the network pharmacology method, as well as revealed the anti-OP effect and potential mechanisms of action of SC.

Author contributions

Wen-jin Liu: Performed the experiments, analyzed the data. Zhengmeng Jiang: Drafted the manuscript. Yi Chen: Conceived and designed the experiments. Ping-ting Xiao: Performed some experiments, analyzed the data. Zi-yuan Wang: Performed some experiments. Tian-qing Huang: Contributed reagents/materials/analysis tools. E-hu Liu: Conceived and designed the experiments, contributed reagents/materials/analysis tools, and wrote and revised the manuscript.

Declaration of competing interest

The authors have declared no conflict of interest.

Acknowledgments

The authors greatly appreciate financial support from the National Natural Science Foundation of China (81973443 and 81922072),

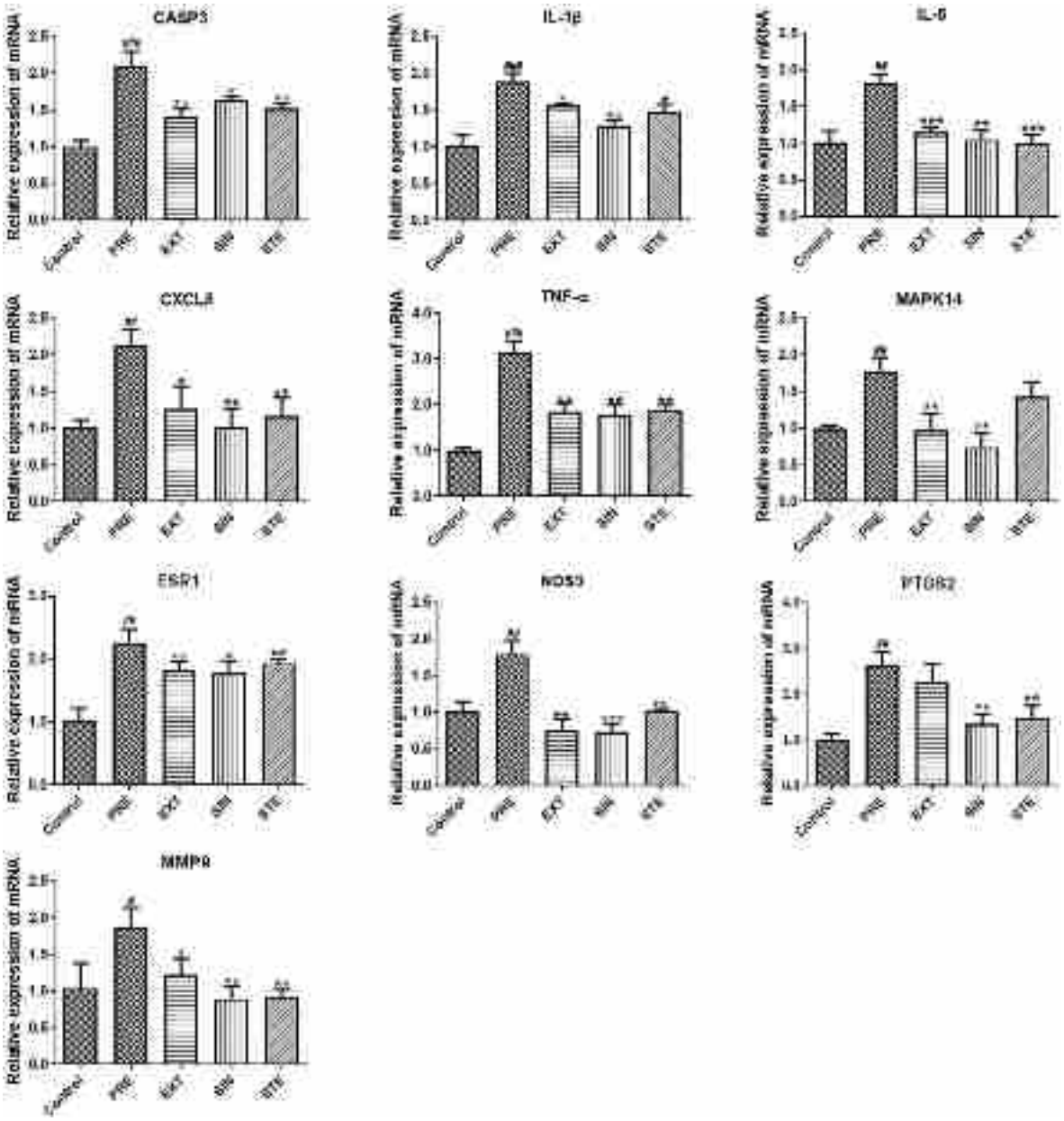

Fig. 6. The expression of OP-related genes in 9-dpf zebrafish larvae after treatment with SC. The selected genes in different experimental groups were determined with quantitative real-time polymerase chain reaction. $^{###}$Compared with Control, $p < 0.001$. *Compared with Pre, $p < 0.05$. **Compared with Pre, $p < 0.01$. ***Compared with Pre, $p < 0.001$.

"Double First-Class" University project (CPU2018PZQ16 and CPU2018GF04) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jep.2020.112871.

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