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In Silico Network Analysis of Ingredients of *Cornus officinalis* in Osteoporosis

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Background: *Cornus officinalis* (CO), also known as 'Shanzhuyu', is one of the most common traditional Chinese herbs used against osteoporosis. Although previous studies have found that CO has beneficial effects in alleviating osteoporosis, its mechanisms remain unclear.

Material/Methods: In this study, we applied system bioinformatic approaches to investigate the possible therapeutic mechanisms of CO against osteoporosis. We collected the active ingredients of CO and their targets from the TCMSP, BATMAN-TCM, and ETCM databases. Next, we obtained the osteoporosis targets from differentially expressed mRNAs from the Gene Expression Omnibus (GEO) gene series (GSE35958). Next, the shared genes of the CO pharmacological targets and osteoporosis-related targets were selected to construct the protein-protein interaction network, based on the results from the STRING database. Subsequently, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were carried out by using the clusterProfiler package in R software.

Results: In all, there were 58 unique CO compounds and 518 therapeutic targets. Based on the GO and KEGG enrichment results of 98 common genes, we selected the top 25 terms, based on the terms' *P* values. We found that the anti-osteoporotic effect of CO may mostly involve the regulation of calcium metabolism and reactive oxygen species, and the estrogen signaling pathway and osteoclast differentiation pathway.

Conclusions: We found the possible mechanisms of CO in treating osteoporosis may be based on multiple targets and pathways. We also provided a theoretical basis and promising direction for investigating the exact anti-osteoporotic mechanisms of CO.

Keywords: **Computational Biology • Medicine, Chinese Traditional • Osteoporosis • Pharmacologic Actions**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/929219>

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Background

Osteoporosis is a systemic bone disease mainly observed in postmenopausal women and the elderly. It is characterized by reduced bone mass and strength and an increased incidence of fractures [1]. Postmenopausal osteoporosis and senile osteoporosis are the 2 main subtypes of primary osteoporosis. In 2016 in China, osteoporosis affected up to 36% of people over the age of 60 years [2]. Currently, it is reported that about 13% of the Chinese population has osteoporosis [3]. Bone fracture is a common complication of osteoporosis, and it was estimated that more than 5.99 million elderly people could have osteoporotic fractures by 2050 in China, with an expected economic cost estimated at 175.4 billion yuan [4,5]. Therefore, it is necessary to take measures to maintain bone mass and lower the incidences of osteoporotic fractures in the population. Clinically, the treatment of the different types of osteoporosis mainly consists of antiresorptive agents and anabolic agents, including calcium, vitamin D, bisphosphonates, and calcitonin [6,7]. However, it is worth noting that these osteoporosis treatments have harmful adverse effects, such as bisphosphonates increasing the risk of breast cancer, jaw necrosis, or even atypical femur fractures [8,9], and excessive calcium intake possibly contributing to the increased risk of cardiovascular events [10]. Therefore, it is important to find safer treatments of osteoporosis. As an alternative to osteoporosis treatment with bisphosphonates or denosumab, an antibody to receptor activator of nuclear factor kappa-B ligand (RANKL), researchers have proposed the use of natural products, such as PMA-zeolite clinoptilolite and calcium-containing zeolite granules, antioxidant compounds such as quercetin and phytoestrogens, and antiresorptive compounds originating from olive oil [11].

In recent years, the development of novel drugs from traditional Chinese medicine (TCM) has attracted increasing attention. TCM possesses the features of having a good curative effect and few adverse effects and thus has been widely used in China for thousands of years. With its satisfactory therapeutic effect and relatively low toxicity, TCM has served as an important alternative treatment in East Asia, Europe, and North America [12,13]. Moreover, kidney tonifying drugs have been widely applied as osteoporosis treatment and have proved effective in treating osteoporosis in experiments [14-16].

Cornus officinalis (CO), also known as 'Shanzhuyu', is one of the effective TCM herbs commonly used in the treatment of osteoporosis. Many studies have confirmed that CO has beneficial effects on alleviating osteoporosis. It was reported that CO can inhibit osteoclast differentiation by suppressing the overexpression of p38 and c-JUN, which is induced by RANKL [17]. Also, the herbal mixture of CO and *Achyranthes japonica* facilitated osteogenesis of MC3T3-E1, inhibited osteoclast differentiation in vivo, and improved the trabecular bone mineral density (BMD)

in an ovariectomy (OVX)-induced osteoporotic mouse model [18]. Similarly, the combination treatment of CO and *Ribes fasciculatum* has the anti-obesity and anti-osteoporotic effects of promoting osteoblastic differentiation and decreasing abdominal fat tissue, respectively. Furthermore, this treatment contributed to the improved BMD and biomechanical properties of an OVX-induced mouse model [19]. Accumulating evidence supports that flavonoids have protective effects against osteoporosis by regulating osteoblast and osteoclast functions [20]. To date, about 20 flavonoids from CO have been found, including quercetin and kaempferol. Quercetin has been extensively investigated in the field of osteoporosis treatment and has been found to suppress osteoclastogenesis and osteoblast apoptosis and to promote osteogenesis and angiogenesis [21]. Iridoids are also essential chemical components of CO; 39 iridoids have been isolated [22], among which morroniside and loganin have been broadly studied. Morroniside and loganin stimulate osteoblast differentiation and indirectly inhibit bone resorption [23].

Although CO has shown potential effects for treating osteoporosis, few studies have comprehensively or systematically investigated its therapeutic mechanism. This may be because CO exerts its therapeutic efficacy through multimolecular balancing in the human body, which is difficult to prove in the scientific experiments widely used in Western medicine [24]. Fortunately, systems pharmacology, an integrity and synergy analysis method, provides researchers a valid tool to explore the therapeutic mechanisms of TCM at a holistic level [25].

In the current study, we utilized an integrated systems pharmacology approach to comprehensively dissect the pharmacological mechanisms of CO in treating osteoporosis. First, we collected the potential active ingredients of CO and their targets through multiple databases. Second, the related osteoporosis targets were obtained from the Gene Expression Omnibus (GEO) data set and then matched with the above CO targets. Third, the protein-protein interaction (PPI) network was constructed on the basis of the common targets from the second step. Finally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were carried out using the clusterProfiler package in R software. Our study provides new insight into the analysis of the therapeutic effects of CO on osteoporosis, based on systems pharmacology and microarray data analysis.

Material and Methods

Screening of Potential Pharmacological Compounds and Targets of CO

We utilized 3 frequently used databases to acquire the therapeutic ingredients of CO, including the Traditional Chinese

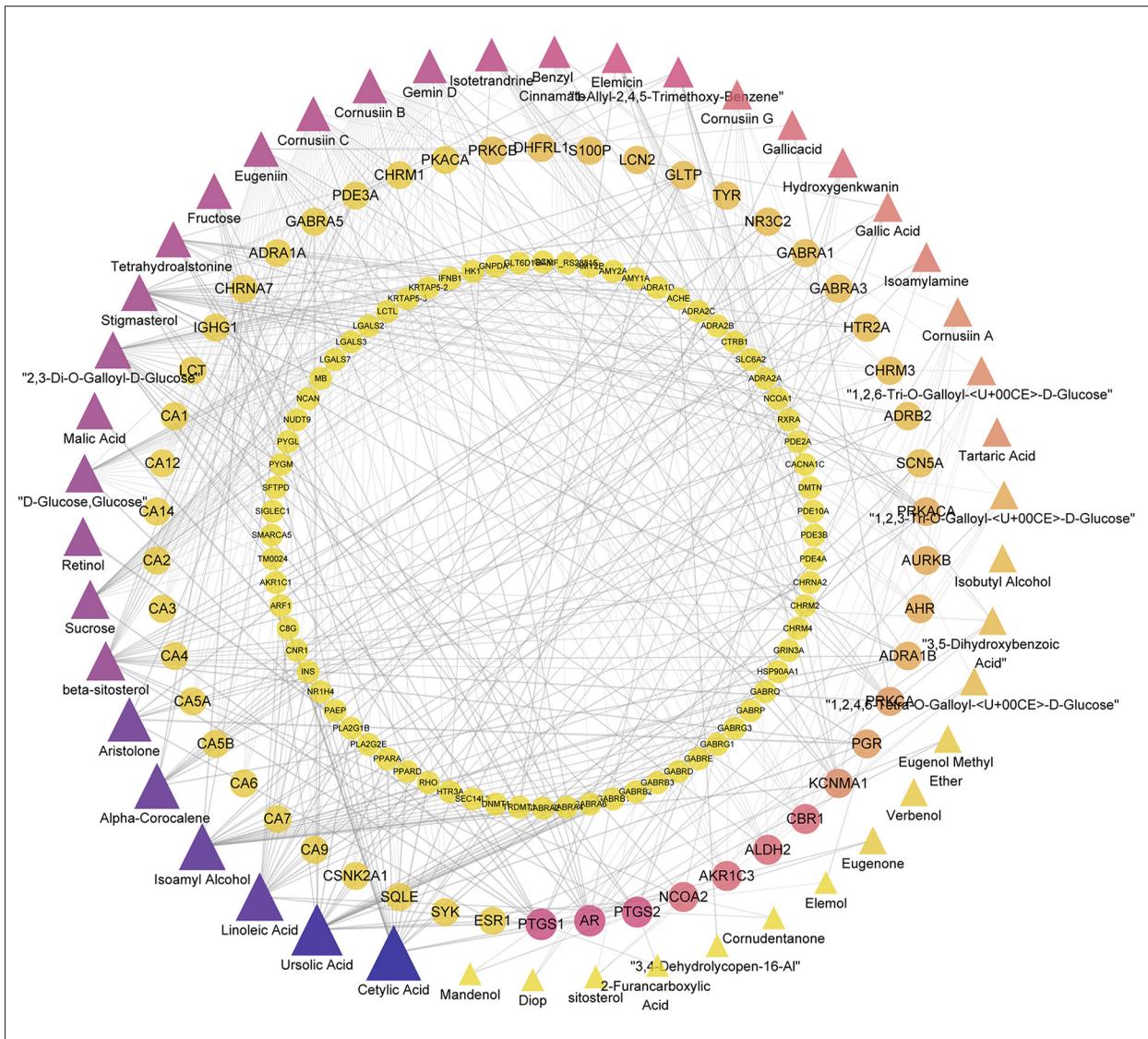


Figure 1. The network for compounds-targets connection. The triangle-shaped nodes are the compounds of *Cornus officinalis* and the round nodes represent their targets. The area and color of all nodes change according to their degree value. Only the nodes with degrees greater than 2 are presented.

Medicine System Pharmacology Database (TCMSP, <http://lsp.nwu.edu.cn/tcmsp.php>) [26]; BATMAN-TCM platform (<http://bionet.ncpsb.org/batman-tcm/>) [27], and the Encyclopedia of Traditional Chinese Medicine platform (<http://www.tcmip.cn/ETCM/index.php/Home/Index/index.html>) [28]. For further compound screening, we set the oral bioavailability threshold at 30% and drug-likeness greater than or equal to 0.18. Oral bioavailability, one of the valid tools in drug screening, signifies the percent of oral administered drugs entered into the body's systemic circulation [29]. Drug-likeness is used to screen out chemically unsuitable compounds and identifies the degree to which a compound is "druglike" [30]. For the BATMAN-TCM database, the thresholds for TAR-score and *P* value cutoffs were set at ≥ 55 and < 0.05 , respectively, to collect

potential ingredients. Moreover, the default cutoff value ≥ 0.8 was used in the ETCM screening part. These ingredients exert their therapeutic functions by interacting with targets; therefore, we further collected the targets predicted by the TCMSP, BATMAN-TCM, and ETCM databases.

The Acquisition of Differentially Expressed Genes in Osteoporosis

We used the GEO database (<https://portal.gdc.cancer.gov/>) to collect osteoporosis-related genes. The terms "osteoporosis" and "OP" were used as search terms, and then the gene expression profile of osteoporosis, named GSE35958, was downloaded. This GEO series contained 4 normal and 5 osteoporotic

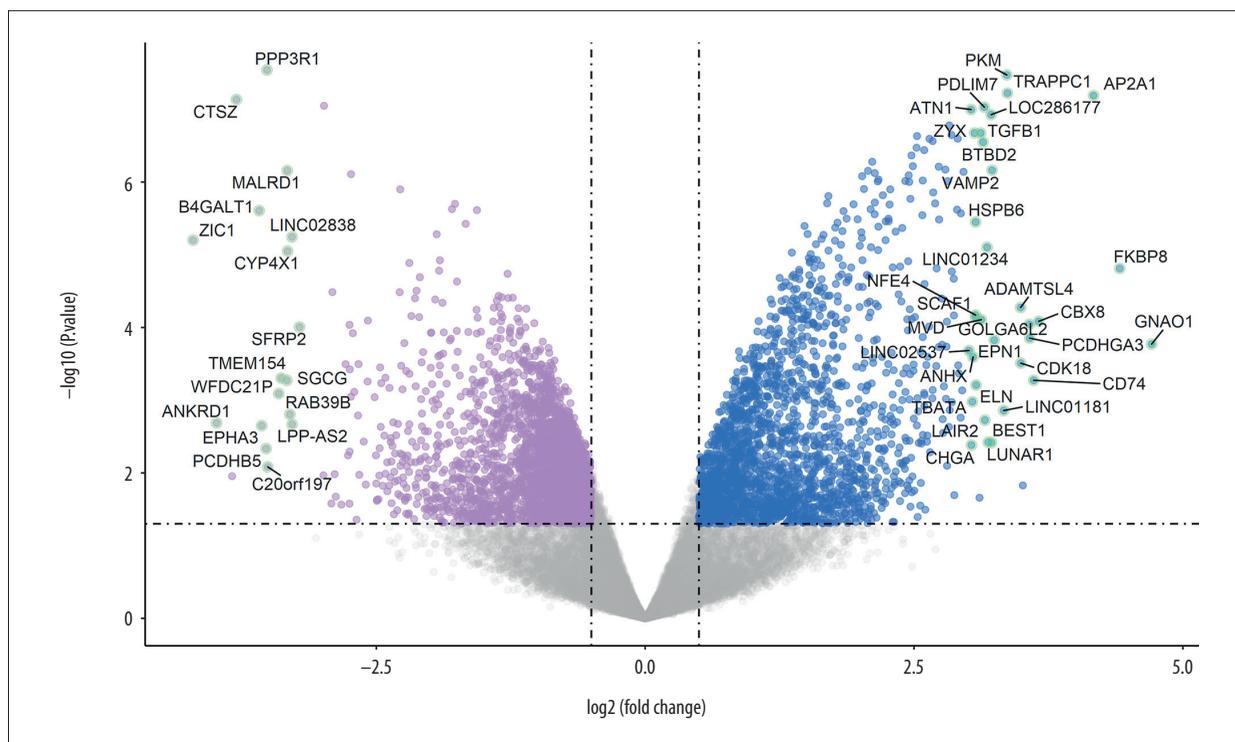


Figure 2. The volcano map to display the gene expression profile of GSE35958. The green dots are the genes with $P < 0.001$ and $|\log_2 \text{fold change}| > 3$.

femoral head bone samples. $P < 0.05$ and $|\log_2 \text{fold change}| > 0.5$ were set as a cutoff threshold to identify differentially expressed mRNAs. Next, the common mRNAs between the normal group and the osteoporosis group were considered as osteoporosis-related genes. The ‘Limma’ package in R software was applied to conduct this process.

Network Construction

The powerful and open-source software Cytoscape (3.7.2, <http://www.cytoscape.org/>) [31] was used to construct 2 critical networks visually, including a compound-target network and PPI network. Next, degree centrality [32], a critical topological parameter, was used to sort nodes in the networks; the greater the number of connections between nodes there were in a network, the more importance a node had.

For the construction of the PPI network, we first drew a Venn diagram using R software (R 3.6.2 for Windows) to obtain the common genes between osteoporosis and potential ingredient targets, which were considered as critical targets when CO exerted its anti-osteoporotic function. Next, the interactions between these intersecting genes were analyzed in the STRING platform [33], and the threshold of the interaction score was set at the highest confidence (0.900) to obtain the most credible interaction. Finally, the exported data of the PPI were used to construct the PPI network in Cytoscape.

GO and KEGG Pathway Enrichment Analysis

After obtaining the common genes, the potential therapeutic targets obtained from GO enrichment (biological process and molecular function) and KEGG pathway enrichment were analyzed through the clusterProfiler package [34] in R. All of these results contributed to exploring the potential biological pathways and functions involved in the anti-osteoporotic function of CO.

Results

Identification of CO Targets

By screening the 3 databases, we collected 14 compounds from TCMSP, 18 from BATMAN-TCM, and 28 from ETCM, yielding 58 unique compounds. Moreover, TCMSP identified 130 bioactive targets, BATMAN-TCM identified 299 targets, and ETCM identified 582 targets. Finally, a total of 518 targets were obtained after taking the union set and deleting duplicate items (Figure 1). In this network, the top 5 compounds were cetyllic acid, ursolic acid, linoleic acid, isoamyl alcohol, and alpha-corocalene.

Potential Targets of Osteoporosis

A total of 4661 differentially expressed genes were detected in the bone samples of patients with osteoporosis, including

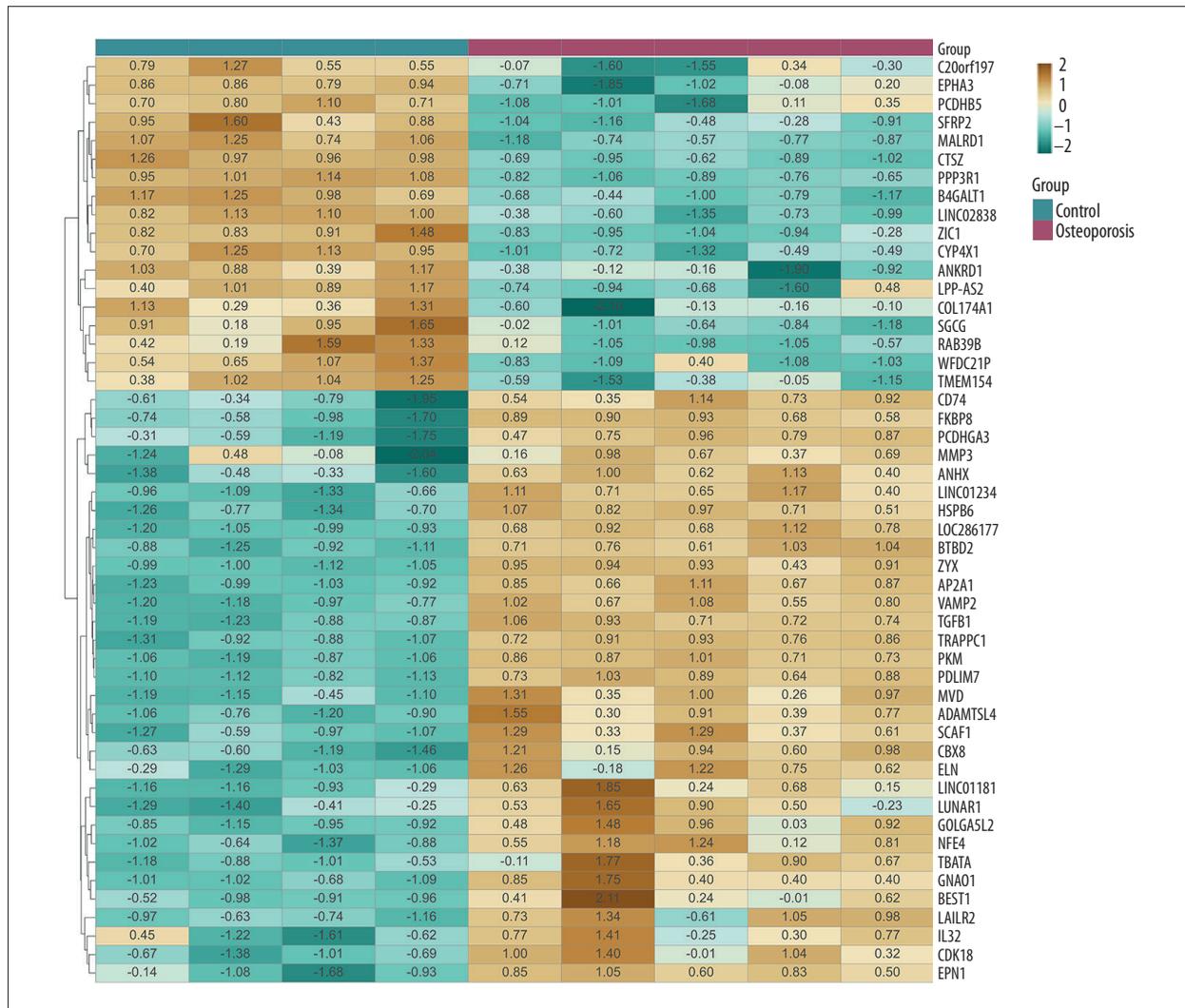


Figure 3. The top 50 differentially expressed genes in GSE35958. The color depth changes are according to their fold change values presented inside the cells

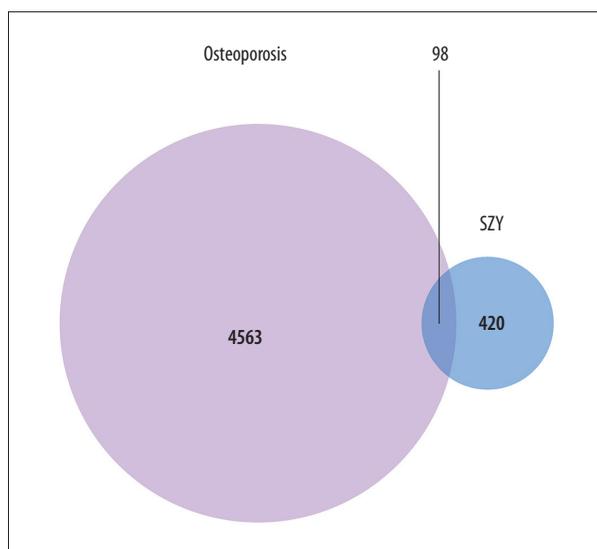


Figure 4. The Venn diagram presents the targets of *Cornus officinalis* and osteoporosis. The overlapping targets are the potential therapeutic genes for *Cornus officinalis* to exert its anti-osteoporotic effect.

2205 upregulated genes and 2456 downregulated genes. We then drew a volcano map to display the gene distribution of the GSE35958 data set (Figure 2). After obtaining the differentially expressed matrix of this data set, we constructed a clustering graph to map the first 50 differential genes sorted by their absolute value of fold change. As shown in Figure 3, the first 4 columns are the control samples and the last 5 are the osteoporotic samples. The changes in color depth are according to the genes' fold changes presented inside the cells.

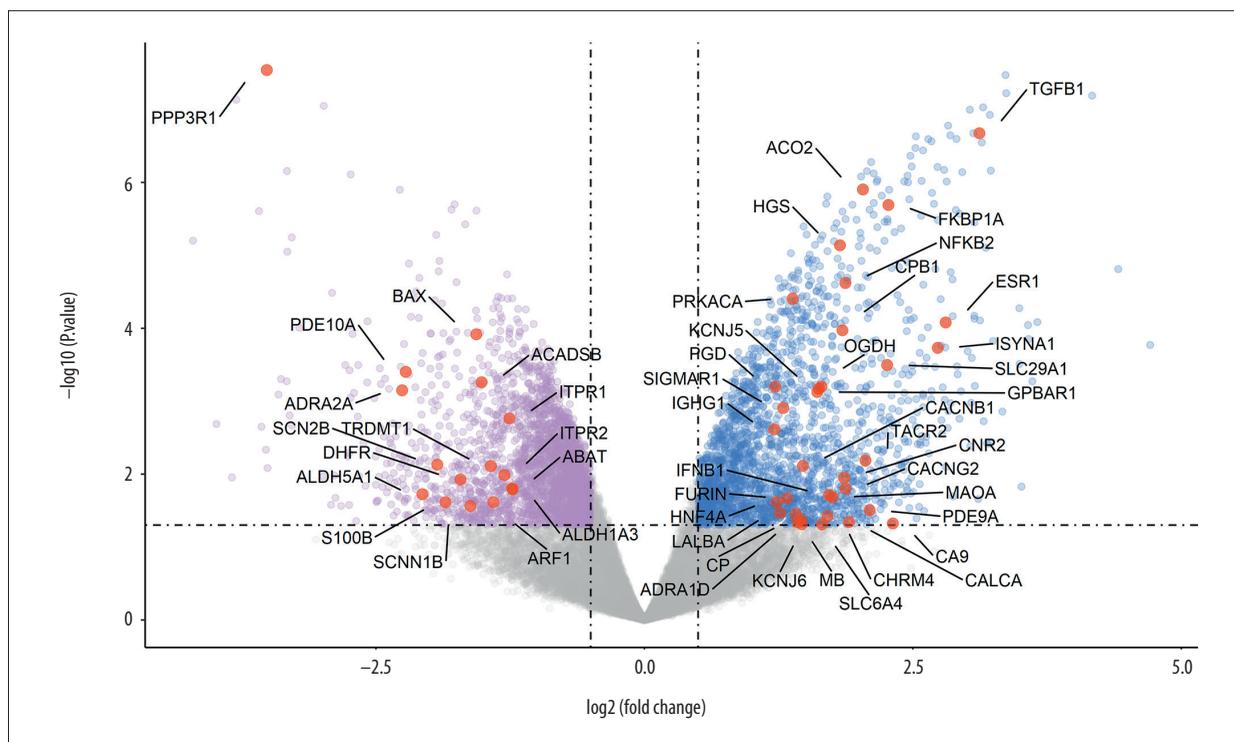


Figure 5. The volcano map displays the gene expression profile of common genes between *Cornus officinalis* and osteoporosis. The red dots are the top 50 genes within 98 therapeutic targets, according to their fold change values.

Intersection Targets and the PPI Network

The 518 predicted targets of CO were intersected with 4661 differentially expressed genes in the osteoporotic bone samples, and 98 therapeutic targets were identified. These key targets may be the therapeutic genes for CO exerting its anti-osteoporotic effect (Figure 4). Subsequently, we determined the expression profile of these 98 genes based on the background of the original chip shown in Figure 5. Among these therapeutic targets, there were 59 overexpressed genes and 39 underexpressed genes. The top 50 common targets are shown in Table 1.

We then obtained the interaction between these common genes based on the result from the STRING database, in which their minimum required interaction score was set to the highest confidence level (0.900). Next, this PPI network, consisting of 52 nodes and 62 edges, was visualized by Cytoscape (Figure 6). Based on the nodes' area, color depth, and location, the genes PIK3R1, PRKCD, ESR1, JUN, IDH1, and ADRA2A may play key roles in this network.

GO Enrichment Analysis

We conducted the GO enrichment of these 98 targets using the clusterProfiler package. A total of 594 biological process terms and 91 molecular function terms were enriched. We sorted

these terms in ascending order of P value and gene count; the top 25 biological process and molecular function terms are shown in Figures 7 and 8. These enrichment results indicated that CO may exert its anti-osteoporotic effect by regulating the transport of the calcium ion transmembrane, release of sequestered calcium ion into cytosol, negative regulation of sequestering of calcium ion, regulation of sequestering of calcium ion, sequestering of calcium ion, and response to reactive oxygen species (ROS).

KEGG Pathway Enrichment Analysis

We then conducted the KEGG pathway enrichment of 98 overlapping genes to determine the possible therapeutic mechanism of CO in osteoporosis. With a cutoff P value of 0.05, a total of 92 pathways were enriched, and then the top 15 pathways were selected based on their P values (Figure 9). Among these pathways, the estrogen signaling pathway (hsa04915) ranked first (P<0.0001) and osteoclast differentiation (hsa04380) ranked twenty-first (P<0.001); these may be the critical signaling pathways for CO in treating osteoporosis.

Discussion

Osteoporosis is a progressive bone disease characterized by decreased bone loss and abnormal bone tissue structure [35].

Table 1. The expression profile of the top 50 common targets between *Cornus officinalis* and osteoporosis.

Gene	logFC	P. value	t	Gene feature
PPP3R1	-3.5165	-17.7179	2.88E-08	Down
TGFB1	3.119761	14.06983	2.11E-07	Up
ACO2	2.034755	11.41889	1.25E-06	Up
FKBP1A	2.272331	10.76941	2.04E-06	Up
HGS	1.821126	9.232075	7.28E-06	Up
NFKB2	1.871123	7.963955	2.39E-05	Up
PRKACA	1.380955	7.47976	3.92E-05	Up
ESR1	2.805662	6.785354	8.32E-05	Up
CPB1	1.843653	6.56717	0.000107	Up
BAX	-1.56528	-6.45756	0.000121	Down
ISYNA1	2.731807	6.096141	0.000185	Up
SLC29A1	2.260512	5.658409	0.000319	Up
SRPK2	-0.88527	-5.49476	0.000393	Down
PDE10A	-2.22243	-5.48864	0.000396	Down
ACADSB	-1.51652	-5.23729	0.000549	Down
OGDH	1.648762	5.13729	0.000628	Up
PGD	1.217724	5.130915	0.000633	Up
KCNJ5	1.624342	5.083025	0.000675	Up
ADRA2A	-2.25607	-5.04617	0.000709	Down
PDE8A	-0.76824	-5.0234	0.000732	Down
GPBAR1	1.610507	5.007926	0.000747	Up
DGKA	1.136045	4.889525	0.000878	Up
PLEKHA1	-0.92004	-4.78965	0.001008	Down
SIGMAR1	1.292762	4.642516	0.001238	Up
HSP90AB1	0.886932	4.550259	0.001411	Up
ITPR1	-1.25692	-4.41186	0.001721	Down
PRKCD	1.153623	4.264664	0.002131	Up
IGHG1	1.210415	4.173964	0.002436	Up
GM2A	0.677983	4.145099	0.002542	Up
PIK3R1	-1.0614	-3.98989	0.003204	Down
PECR	-1.10672	-3.91403	0.003593	Down
ACSL4	-0.97685	-3.89446	0.003701	Down
GABRG1	1.176707	3.840374	0.004018	Up
CHRNA2	0.672786	3.809501	0.004212	Up
LTB4R	0.944593	3.794815	0.004307	Up

Table 1 continued. The expression profile of the top 50 common targets between *Cornus officinalis* and osteoporosis.

Gene	logFC	P. value	t	Gene feature
ABL1	0.792837	3.672926	0.005195	Up
FTH1	0.579314	3.648804	0.005392	Up
TACR2	2.058494	3.528729	0.0065	Up
NCOA2	-1.03617	-3.51378	0.006654	Down
MAN2A1	-0.75873	-3.4979	0.006821	Down
PRKAA1	-1.17363	-3.48005	0.007015	Down
SCN2B	-1.92859	-3.44509	0.007411	Down
CACNB1	1.475235	3.419732	0.007712	Up
TRDMT1	-1.42989	-3.41586	0.00776	Down
ACSL3	-0.61941	-3.40479	0.007896	Down
PRKDC	-1.11193	-3.37398	0.00829	Down
HOXA10	-0.70229	-3.27324	0.009726	Down
GSTP1	0.703063	3.267906	0.009809	Up
HPRT1	-0.59404	-3.24227	0.010218	Down
ITPR2	-1.30336	-3.23764	0.010294	Down

With the aging of society worldwide, osteoporosis is a common disease threatening this population; osteoporosis also imposes a significant economic burden on healthcare systems [36]. Therefore, it is economical and desirable to alleviate osteoporosis and its related fractures [37]. The loss of bone mass resulting from osteoporosis is caused by the lack of balance between bone absorption, which is determined by osteoclasts, and bone formation, which is determined by osteoblasts [38]. Bone resorption inhibitors are currently the primary treatment for osteoporosis. However, the available bone resorption drugs have serious adverse effects associated with long-term use, such as cardiovascular events, atypical long bone fractures, and jaw osteonecrosis [39,40]. Therefore, there is a practical need to find alternative and safer treatments capable of protecting against osteoporosis [41].

CO helps prevent osteoporosis by prohibiting osteoclast activity and promoting osteogenic differentiation, which may be associated with its regulating effects on related targets. Therefore, we applied the network pharmacology method to explore the potential mechanism of CO at a systems level. Through the compounds-targets network (Figure 4), we found that the top 5 ingredients of CO, cetyllic acid, ursolic acid, linoleic acid, isomyl alcohol, and alpha-corocalene, may play critical roles in this network. Therefore, the promising anti-osteoporotic effects of CO may be closely related to these compounds. Lee et al found, through in vitro and in vivo experiments, that ursolic

acid enhances osteoblast differentiation and promotes bone formation [42]. Also, ursolic acid suppresses osteoclast differentiation and inhibits osteoclast-induced bone loss, which may be related to the NF-κB signaling pathway [43]. Currently, there is no literature available about the effects of linoleic acid on osteoclasts or osteoblasts. However, a study showed that linoleic acid, as the agonist for peroxisome proliferator activator receptors, can significantly improve osteoblast differentiation and increase serum bone formation markers and BMD in male rats [44]. Moreover, conjugated linoleic acid, the isomer of linoleic acid, possesses the promising regulatory ability of osteoclast and osteoblast differentiation [45]. Although some compounds, such as morroniside and loganin, have been extensively studied in the field of osteoporosis [23], they were not involved in the present study owing to their low oral bioavailability or drug-likeness values, which are critical pharmacokinetic tools for drug screening. Therefore, we will prioritize compounds such as ursolic acid in further studies on the regulatory effects of CO on osteoclastic and osteoblastic differentiation.

A Venn graph between CO targets and differentially expressed genes of osteoporosis identified 98 common genes as therapeutic targets, which may play critical roles in CO exerting its anti-osteoporotic effect. Indeed, some clinical trials and animal experiments found that these genes have anti-osteoporotic effects. Transforming growth factor-β1 (TGF-β1) is a

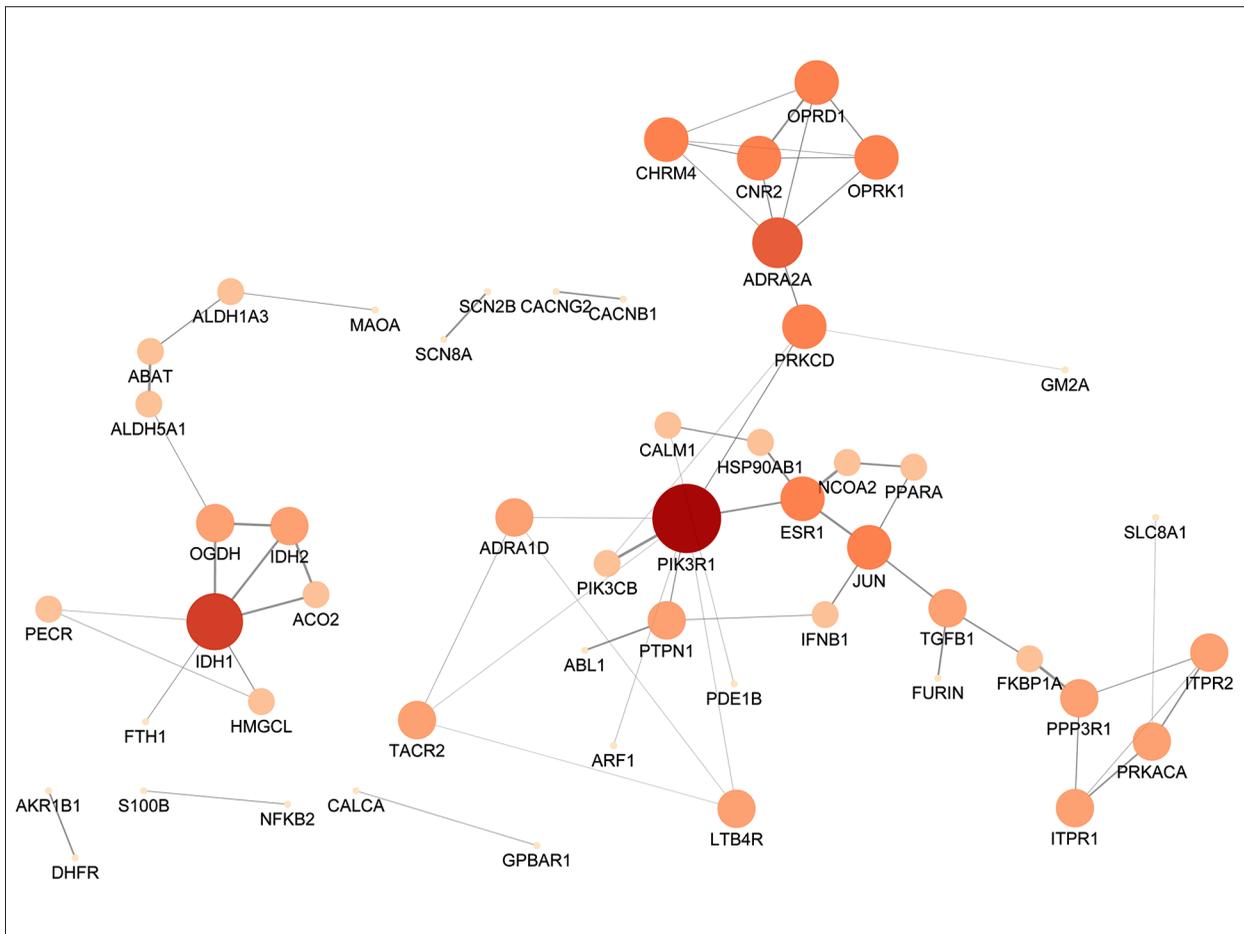


Figure 6. The protein-protein interaction network of 98 candidate targets of *Cornus officinalis* in treating osteoporosis. The area and color of all nodes change according to their degree value.

highly expressed bone matrix protein that regulates interactions between osteoclasts and osteoblasts to balance bone remodeling, thereby maintaining sufficient bone mass. It has been shown that the polymorphism of the TGF- β 1 gene has an adverse effect on BMD and increases the susceptibility of osteoporosis [46]. Similarly, Zhou et al used peripheral blood monocytes for a cell model and confirmed that NFKB2, via exon arrays, was differentially expressed in high and low BMD groups [47]. Moreover, 2-sample mendelian randomization confirmed that the ESR1 gene influenced BMD and was therefore associated with fracture risk [48]. Wang et al also found that ESR1 is a key risk factor for osteoporotic fracture in Chinese postmenopausal women [49]. PIK3R1, a key regulatory factor of the PI3K/Akt signaling pathway, plays a critical role in alleviating osteoporosis [50,51]. Kim et al showed that cells laced with Bax attenuate osteoclast apoptosis [52].

According to the drug theory of “multi-ingredient, multitarget” [53], the above results showed that the multiple active ingredients of CO may interact with different genes to exert its anti-osteoporotic effect. Therefore, we further conducted

GO and KEGG pathway enrichment to analyze the functions of these genes. GO is a collection of controlled vocabularies for gene functions, and many genomes have been annotated with GO terms, including biological process, cellular component, and molecular function. Therefore, GO enrichment analysis finds GO terms that are abnormally expressed for a given set of interesting genes, enabling their biological interpretation. Moreover, KEGG pathway enrichment demonstrates additional information about the patterns of genes interacting in pathways [54]. After conducting these 2 enrichments based on 98 common genes, we suggested that the therapeutic effect of CO in osteoporosis treatment may involve the regulation of calcium metabolism and ROS biological processes and the estrogen signaling and osteoclast differentiation pathways.

The differentiation of osteoclasts and osteoblasts and their proliferation are all closely related to ROS [54,55]. ROS can cause serious damage to periodontal tissue through accelerating bone resorption, which is closely related to the upregulated osteoclast differentiation by NF- κ B and the calcineurin-NFAT pathway [56,57]. We found several GO biological process

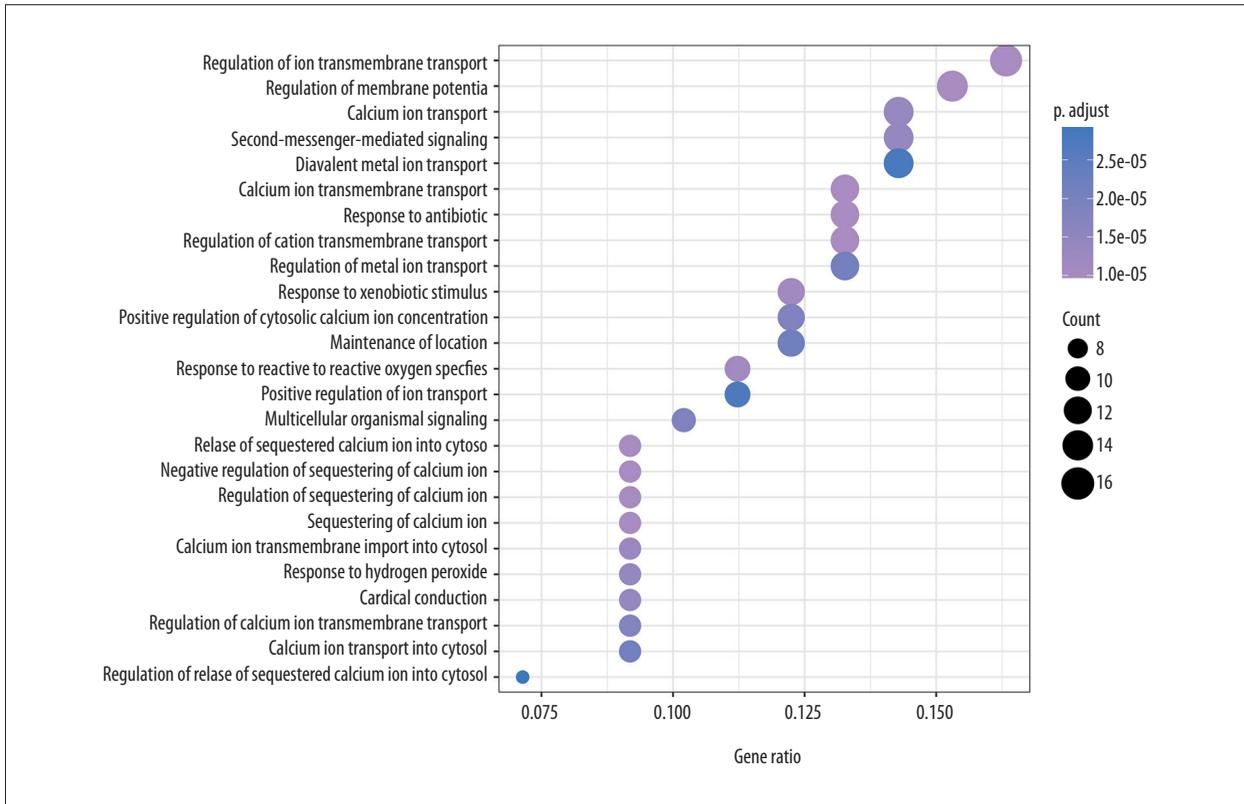


Figure 7. The top 25 processes of the biological process enrichment.

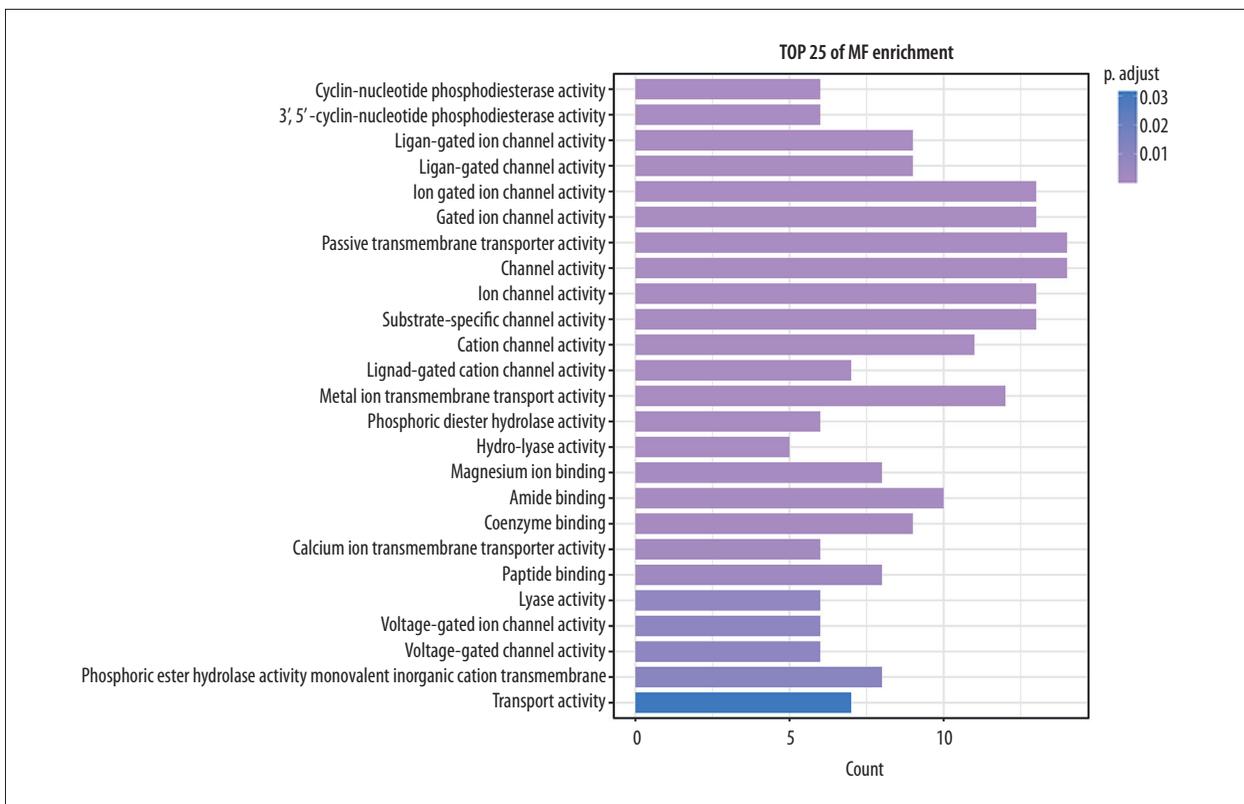


Figure 8. The top 25 processes of the molecular function enrichment.

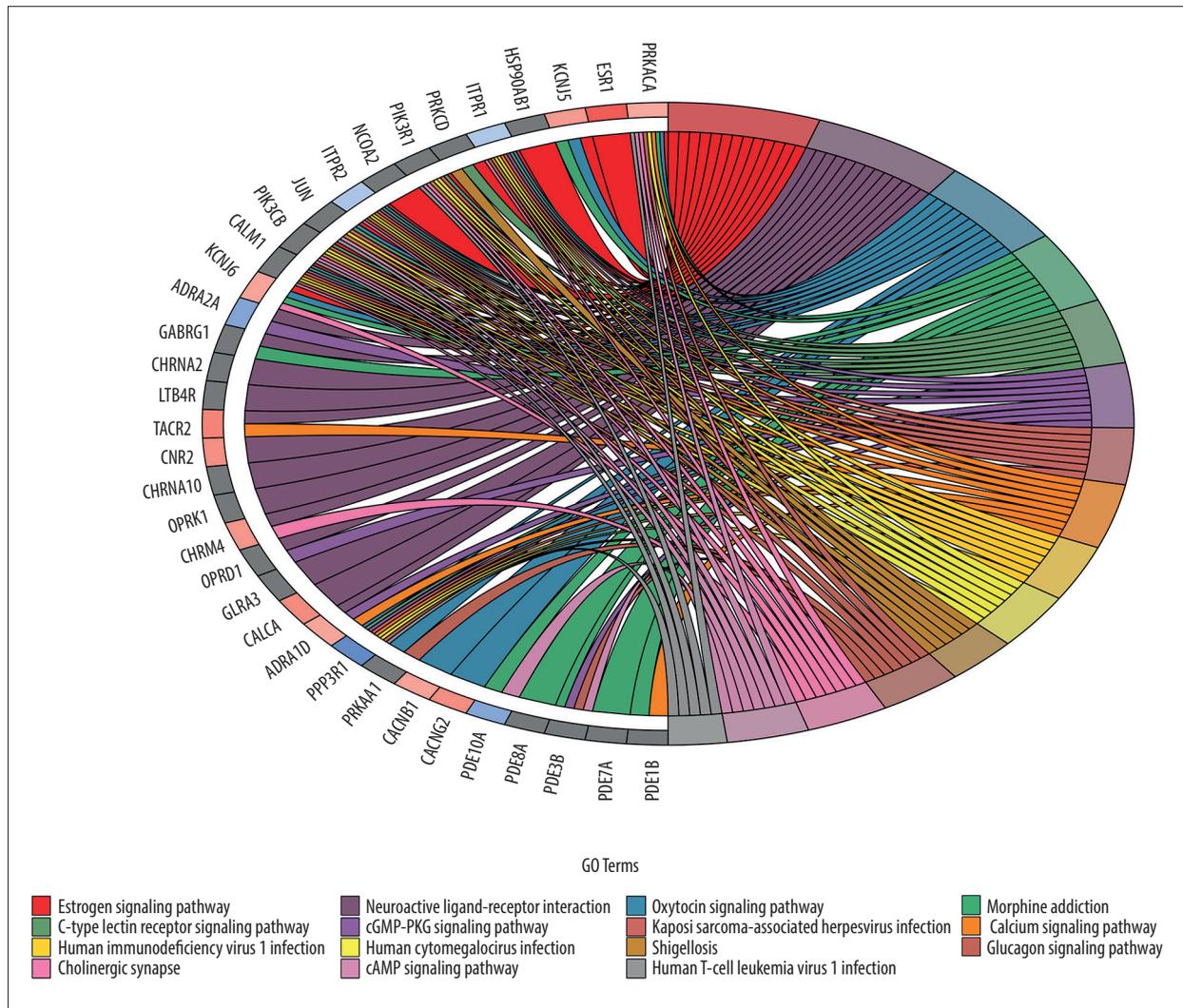


Figure 9. Chord diagram for the top 15 Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment terms.

terms are associated with reactive ROS, including GO: 0000302 (response to ROS), GO: 0034614 (cellular response ROX), GO: 0006979 (response to oxidative stress), and GO: 0034599 (cellular response to oxidative stress). Although there are no studies on the anti-ROS effect of CO compounds on osteoblasts or osteoclasts, several studies confirmed that CO compounds, such as ursolic acid and linoleic acid, can reduce ROS production in cancer cells [55,56]. Hwang et al showed that the ethanol extract of CO can protect RAW 264.7 cells from lipopolysaccharide-induced oxidative stress [57]. Therefore, it is worth validating the anti-ROS effects of CO compounds on bone cells in the future.

In addition, the anti-osteoporotic effect of CO may be associated with the regulation of calcium ions; the top 5 related GO biological process terms were GO: 0051209 (release of sequestered calcium ion into cytosol), GO: 0051282 (regulation of sequestering of calcium ion), GO: 0051208 (sequestering of

calcium ion), GO: 0097553 (calcium ion transmembrane import into cytosol), and GO: 0006816 (calcium ion transport). Calcium supplementation is a critical part of the prevention and treatment of osteoporosis. Increasing evidence shows that aging is associated with a disturbed calcium balance. Heshmati et al found that, in addition to estrogen deficiency, women with osteoporosis who are postmenopausal have a parathyroid hormone defect, which leads to a negative calcium balance [58]. Ursolic acid has been found to promote calcium balance and have an inhibitory effect on the age-induced secondary hyperparathyroidism of female rats, which may explain the anti-osteoporotic effect of ursolic acid [59]. Linoleic acid is the main source of n-6 fatty acids, which are able to regulate calcium balance, osteoblastogenesis, and osteoblast activity [60]. Gallic acid and β -sitosterol have also been shown to have osteogenic activity and a promotional effect on serum calcium and ALP in an OVX-induced rat model [61]. Moreover, our KEGG enrichment analysis revealed that the calcium signaling pathway

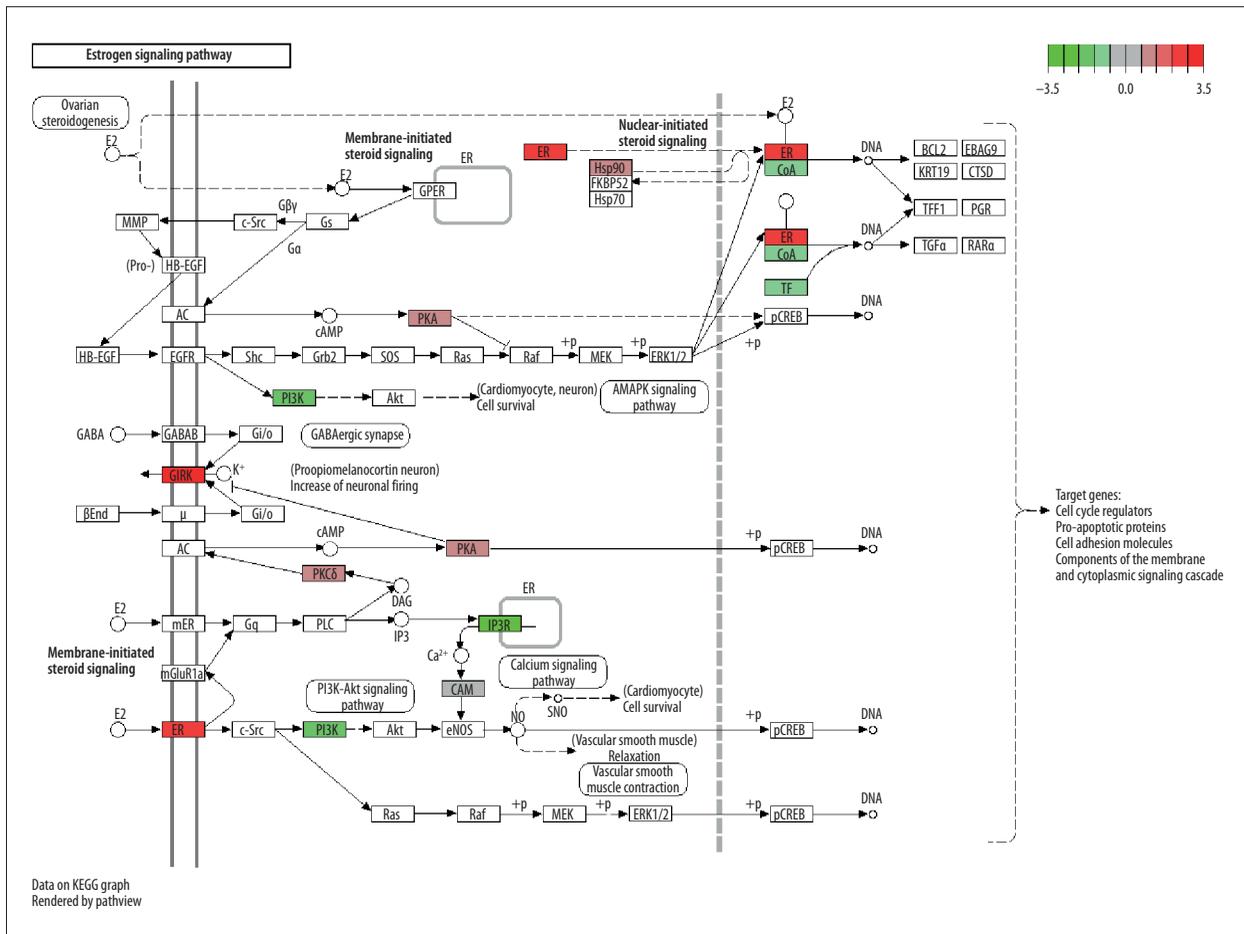


Figure 10. The estrogen signaling pathway ranked first in Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment terms.

(hsa04020) is one of the top 25 pathways. Therefore, the anti-osteoporotic ability of CO may be associated with calcium balance; therefore, the calcium regulatory effect of other compounds deserve further investigation.

In terms of KEGG enrichment, the estrogen signaling pathway (hsa04915) ranked first, which means this pathway plays a critical role in CO's ability to treat osteoporosis (Figure 10). Deficient estrogen is a critical cause of osteoporosis, and estrogens can alleviate bone loss through decreased osteoclastogenesis [62]. Recently, Park et al showed the anti-menopausal effects of CO can promote the expression of ESR1 mRNA, one of the common genes from our results, and statistically significantly improve the BMD of OVX-induced mice [62]. Conjugated linoleic acid has been found to attenuate bone loss in OVX-induced mice resulting from estrogen deficiency [45]. Gallic acid and β-sitosterol can also ameliorate the consequences of OVX due to estrogenic modulation [61]. Retinol, a commonly used vitamin, can intensify the bone constructs and bone mass in estrogen deficient rats [64].

Moreover, based on the result of our present KEGG enrichment analysis, CO may have a regulating role in the osteoclast

differentiation pathway (hsa04380). Six common genes, TGFB1, NFKB2, JUN, PPP3R1, PIK3R1, and IFNB1, were enriched in this signaling pathway (Figure 11). These genes have been previously reported to promote osteoclastogenesis [65-67]. Urosolic acid was found to suppress NF-κB signaling and c-Jun N-terminal kinase signaling [43]. A recent study also indicated that ursolic acid negatively regulates PI3K/AKT signaling, inhibiting TGF-β1-induced human endothelial cell fibrosis [68]. Urosolic acid can also reduce phosphorylation of nuclear factor κB kinase subunit β and IκBα, as well as suppress the activation of NF-κB and MAPKs of macrophages under the stimulation of lipopolysaccharide [69]. A previous study demonstrated that linoleic acid can reduce the expression levels of P-mTOR, P-Akt, and P-PI3K proteins in RAW 264.7 cells [70].

The present study has limitations and aspects that can be improved in the future. We discussed only a small proportion of potential anti-osteoporotic mechanisms for CO. Because of inadequate funding, the blood compositions of CO were not confirmed through mass spectrometry, which may have caused certain errors. Moreover, we lacked in vivo and in vitro experimental validation, thereby limiting the reliability of the current

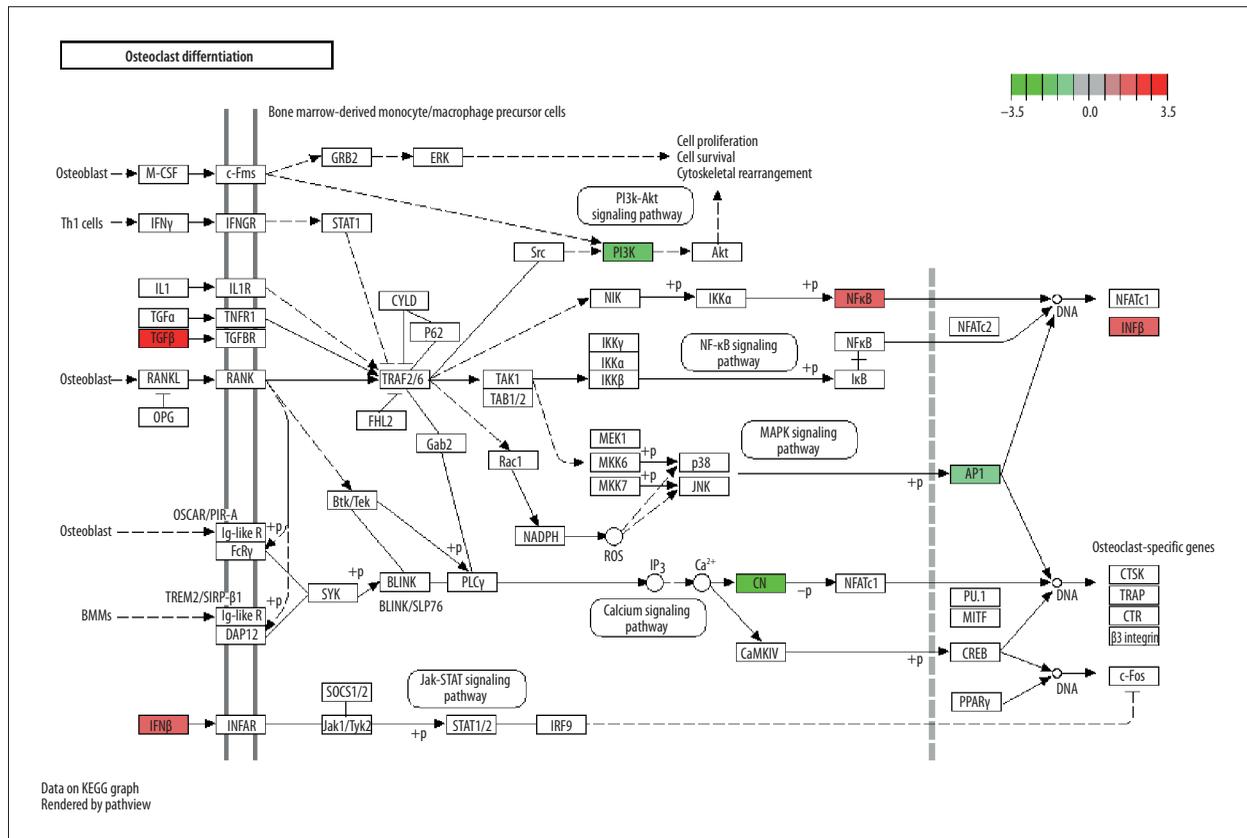


Figure 11. The osteoclast differentiation signaling pathway ranked twenty-first in Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment terms.

results. However, our results still provided us with promising compounds and pathways for prioritizing future research.

Conclusions

The present study illustrated that the potential targets and underlying mechanisms of CO in treating osteoporosis may occur through regulating ROS and calcium metabolism. Based on the KEGG enrichment results, we determined that these anti-osteoporotic effects may be mainly involved with the estrogen signaling pathway and osteoclast differentiation pathway. However, because the present results were generated from data mining and data analysis, *in vitro* and *in vivo* experiments should be conducted in the future.

Conflicts of Interest

None.

Supplementary Data

All data used in the present study have been uploaded to the Figshare repository (<https://doi.org/10.6084/m9.figshare.13017911.v1>).

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