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Systems pharmacology dissection of action mechanisms of Dipsaci Radix for osteoporosis

Wenjuan Zhang[1], Kaiyue Xue[1], Yongguang Gao[1], Ying Huai, Wei Wang, Zhiping Miao, Kai Dang, Shanfeng Jiang, Airong Qian*

Lab for Bone Metabolism, Key Lab for Space Biosciences and Biotechnology, School of Life Science, Northwestern Polytechnical University, Xi'an, Shaanxi 710072, China
Research Center for Special Medicine and Health Systems Engineering, School of Life Science, Northwestern Polytechnical University, Xi'an, Shaanxi 710072, China
NPU-UAB Joint Laboratory for Bone Metabolism, School of Life Science, Northwestern Polytechnical University, Xi'an, Shaanxi 710072, China

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ABSTRACT

Aims: Osteoporosis (OP) is a systemic metabolic bone disease characterized by bone mass decrease and microstructural degradation, which may increase the risk of bone fracture and leading to high morbidity. *Dipsaci Radix* (DR), one typical traditional Chinese medicine (TCM), which has been applied in the treatment of OP with good therapeutic effects and few side effects. However, the underlying molecular mechanisms of DR to treat OP have not been fully elucidated. In this study, we aim to dissect the molecular mechanism of DR in the treatment of OP.

Materials and methods: A systems pharmacology approach was employed to comprehensively dissect the action mechanisms of DR for the treatment of OP.

Key findings: 10 compounds were screened out as the potential active ingredients with excellent biological activity based on in silico ADME (absorption, distribution, metabolism and excretion) prediction model. Then, 36 key protein targets of 6 compounds were identified by systems drug targeting model (SysDT) and they were involved in several biological processes, such as osteoclast differentiation, osteoblast differentiation and anti-inflammation. The target-pathway network indicated that targets are mainly mapped in multiple signaling pathways, i.e., MAPK, Tumor necrosis factor α (TNF-α), NF-κb and Toll-like receptor pathways. The in vitro results indicated that the compounds ursolic acid and beta-sitosterol effectively inhibited the osteoclast differentiation.

Significance: These results systematically dissected that DR exhibits the therapeutic effects of OP by the regulation of immune system-related pathways, which provide novel perspective to drug development of OP.

1. Introduction

OP is a systemic metabolic bone disease characterized with decreased bone mineral density (BMD) and increased risk of bone fragility, which has become one of the important public health issues for the elderly population and postmenopausal women [1–3]. Increasing attention has been paid to OP in aged worldwide because it may lead to an increased risk of fractures [4–7]. In China, with the increasing aging population, the incidence of OP and osteoporotic fracture incidence increased quickly [8]. It is reported that the prevalence of OP was 36% over 60 years old in 2016. It is estimated that by 2050 in China, > 5.99 million aged people may develop into osteoporotic fractures, with a direct economic cost of approximately 175.4 billion yuan [9–11]. More importantly, osteoporotic fractures may result in chronic pain and disability, which are seriously threatening the human health, and even leading to death [12,13]. Therefore, it is urgent to develop effective therapeutic strategy for the intervention and treatment of OP.

Clinically, a number of drugs are commonly used for the treatment of OP including calcium (Ca), vitamin D, bisphosphonates and calcitonin [14–18]. In addition, the excessive calcium intake increased risk for cardiovascular events, urolithiasis and even fractures [19]. In fact, the efficacy of available drugs is not so satisfactory and may cause side effects, such as the bisphosphonates may increase the risk of cardiovascular diseases [15,20]. Hence, a more effective and less side effect strategy is needed in the novel drug development for the treatment of OP. In recent years, more attention has been drawn to developing novel

* Corresponding author at: 127 Youyixi road, Xi'an City, Shanxi Province, China.
E-mail address: qianair@nwpu.edu.cn (A. Qian).
[1] Equal contributors.

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drugs from TCM, which were holistic systems with good therapeutic effects and few side effects, and was widely used in clinical practice over thousands of years. Actually, kidney-tonifying traditional Chinese medicine is always widely used for the treatment of OP [21].

DR is a classical kidney-tonifying medicine, derived from the dry roots of *Dipsacus asper Wall. ex Henry (Dipsacaceae)*, with a long history of safe use for the treatment of OP and bone fractures [22–25]. Also, DR is one of the common herbal tea beverages or food diet for the medical applications. When it was consumed in the form of tea, it also has benefits in cases of backache, legs sore and bone diseases. Moreover, DR has widespread acceptance as a food diet for strengthening liver and kidney and alleviating the backpain and edema, whereas its therapeutic value is criticized for a lack of systematic analysis, yet have not accepted by mainstream medicine.

A growing body of evidences have highlighted that DR possesses the beneficial roles on increasing bone density and bone strength [21,26,27]. It was reported that DR extract taken orally increased bone density and altered bone histomorphology, thus exhibiting the good potential for treatment of postmenopausal OP [28–30]. In addition, many modern medical studies have confirmed that DR effectively reduced the bone turnover rate and reestablish the dynamic balance between bone formation and bone resorption [26,31]. However, it was apparent that few researches comprehensively and systematically dissected the potential active compounds and the action mechanisms of DR.

Here, an integrated systems pharmacology approach was used to dissect the action mechanisms of DR for treating OP from a systematic level (Fig. 1). First, the potential active ingredients of DR with favorable pharmacokinetic properties were screened out by a system-ADME model. Second, the protein targets of these bioactive compounds associated with OP were identified by SysDT model and database searching. Third, the crucial disease-relevant biological processes were obtained by the GO analysis. Finally, the analysis of active component-target network and target-pathway network were implemented to interpret the multi-mechanisms of DR. Our study provides a new insight into the dissection of the action mechanisms of DR in treating OP, offering a promising strategy for novel drug discovery from herbal medicine.

2. Materials and methods

2.1. Ingredient database construction of DR

All compounds of DR were obtained from TCMSP database (http://lsp.nwsuaf.edu.cn/tcmsp.php) [32]. Then, the chemical structures of these ingredients were downloaded from Pubchem (https://www.ncbi.nlm.nih.gov/pccompound/) and Chemical book databases (https://www.chemicalbook.com/). Due to the deglycosylation of glycosides by colonic bacteria in humans, the corresponding aglycones of the ingredients were also retained for following studies.

2.2. Active ingredient screening

To obtain the potential pharmacological active compounds of DR, three ADME screening models, i.e., PreOB (predict oral bioavailability) model, PreDL (predict drug-likeness) model and Caco-2 model were employed to prescreen the potential bioactive molecules. In detail, PreOB, a potent in-house previously developed model [33] was employed to predict the oral bioavailability (OB) of the constituents of the herb. Furthermore, to obtain drug-like compounds, PreDL [34], a database-dependent model, was also carried out to compute the drug-likeness of each compound by using the Tanimoto coefficient (as displayed in Eq. (1)).

$$T(A,B) = \frac{A \bullet B}{\|A\|^2 + \|B\|^2 - A \bullet B} \quad (1)$$

where A shows the molecular properties of herbal ingredients, and B displays the average molecular properties of molecules in DrugBank database (http://www.drugbank.ca/) based on Dragon soft descriptors. Since the average DL value of all 6511 molecules in DrugBank is 0.18, the molecules with DL larger than 0.18 were considered as with good drug-likeness.

Also, Caco-2 was an important parameter for stimulation of drug transport across the monolayer of small intestinal epithelial cells, which was crucial for the prediction of drug absorption [35]. In the study, the Caco-2 ≥ -0.4 were considered as the candidate active compound. Finally, the molecules with OB $\geq$ 30%, DL $\geq$ 0.18 and Caco-2 ≥ -0.4 were selected as candidate compounds for further analysis.

2.3. Target fishing of OP

To identify targets of these potential active ingredients, an in-house developed model SysDT (systematic drug targeting model) based on RF and SVM methods was proposed, which efficiently integrated large scale information of chemistry, genomics and pharmacology [36]. Then, to obtain the targets associated with OP, they were identified from three databases including Target Database (TTD, http://bidd.nus.edu.sg/group/ttd/) [37], the Human Gene Database (GeneCards), DrugBank and the HIT database (Herbal Ingredients' Targets Database, http://lifecenter.sgst. cn/hit/) [38].

2.4. GO analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resource comprises an integrated online biological knowledge base and analytical tools, which are applied for systematically extracting biological data from large gene and protein lists (http://david.abcc.ncifcrf.gov/) [39]. In this study, to identify the biological processes of the predicted targets involved in, the targets were mapped into DAVID . Threshold count $\geq$2 and EASE score $\leq$0.05 were selected in functional annotation clustering.

2.5. Network construction and analysis

To characterize the multi-compound therapeutic features of DR, two networks were constructed: (1) the compound-target (C-T) network, based on the obtained candidate compounds and their potential targets; (2) the target-pathway (T-P) network, based on the potential targets and the signaling pathways in which they participated in. The networks were constructed by Cytoscape 3.6.0 software [40]. In these bilateral networks, the nodes represent the compounds, potential targets, or signal pathways, and the edges represent the interactions between compound, target and pathway.

2.6. Docking analysis

To further investigate the mechanism of the interactions between the candidate active compounds and targets, four key targets of OP in C-T network were selected for docking simulations. The docking simulation was performed by molecular docking program GOLD (version 5.1). The X-ray crystal structures of four targets Apoptosis regulator Bcl-2 (BCL2), Interleukin 6 (IL-6), Transcription factor AP-1 (JUN) and apoptosis regulator BAX (BAX) were extracted from RCSB Protein Data Bank. The binding site was defined as the volume of co-crystalized ligands in the template proteins. The radius around the ligand was set 10 Å in the crystal structure as the binding pocket and the 10 top-ranked docking poses were selected for further analysis.

2.7. The experimental validation

To further validate the therapeutic effects of the candidate compound for OP, the compounds ursolic acid and beta-sitosterol (the purity > 98%) were selected for the in vitro experiments. Both

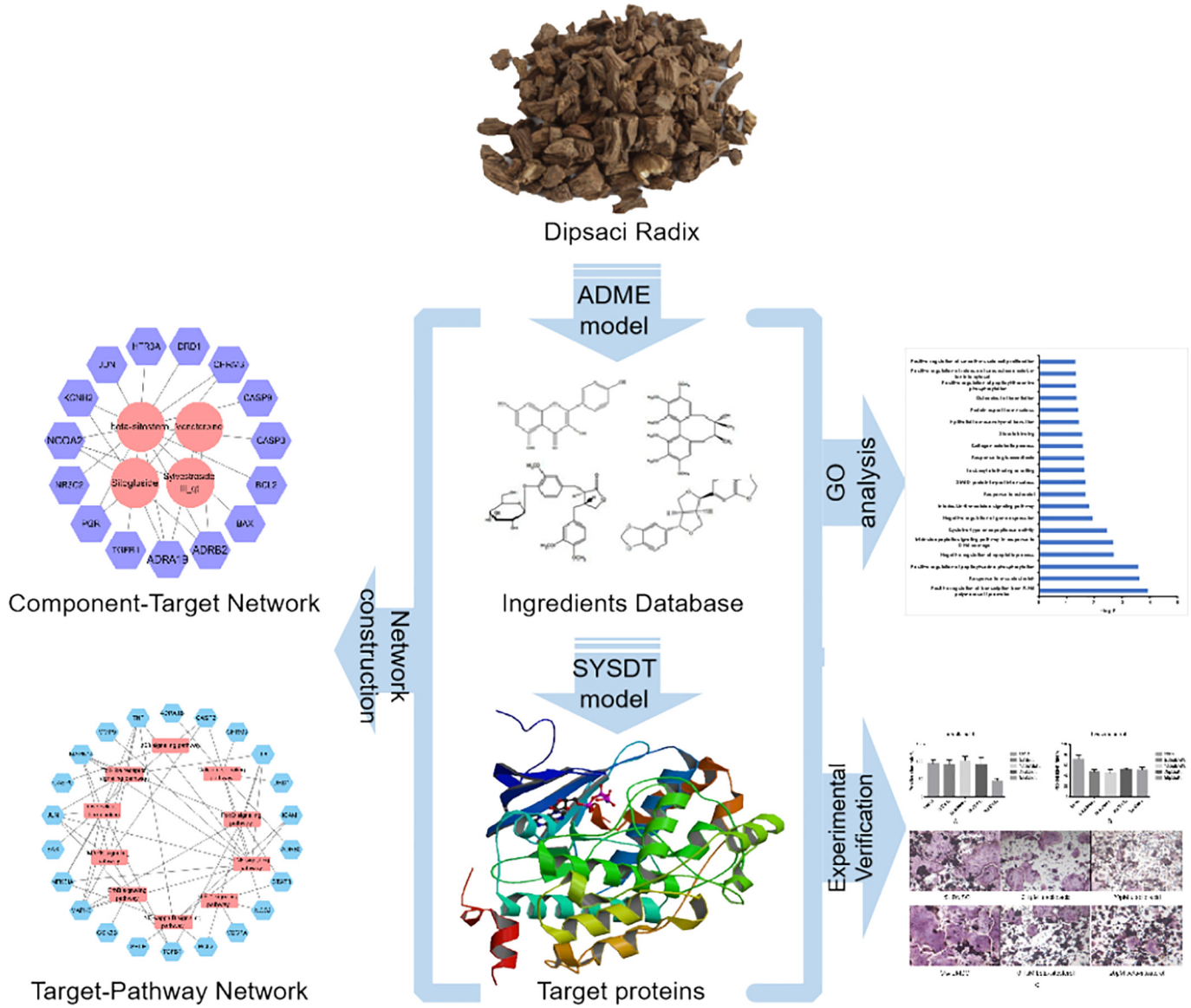


Fig. 1. The work scheme of systems pharmacology approach. ADME: absorption, distribution, metabolism and excretion; SYSDT: systematic drug targeting model.

compounds were purchased from Chroma Biotechnology Co. Ltd. (Chengdu, China). The cell proliferation of compounds ursolic acid and beta-sitosterol was performed by MTT method. Osteoclast cell line Raw 264.7 cells were incubated in DMEM complete media supplemented with 10% fetal bovine serum, 100 U/ml penicillin. To investigate the effect of candidate compounds on the function osteoclast, RAW 264.7 (1.5×10^4 cells/well) were cultured in complete medium containing RANKL (50 ng/ml) in a 48-well (300 ml/well) plate with or without ursolic acid and beta-sitosterol. After 4 days, cells were fixed in 4% paraformaldehyde solution for 20–30 min, and then stained with tartrate-resistant acid phosphatase (TRAP) using the Acid phosphatase test kit.

Acid phosphatase test kit was purchased from Sigma Aldrich (Cat. No.: 387A-1KT). Recombinant mouse RANKL (E.coil-expressed) were purchased from R&D, USA. BCIP/NBT Alkaline Phosphatase Color Development Kit were purchased from Beyotime (Shanghai, China). β-Glycerophosphate sodium and ascorbic acid were from American.

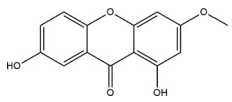
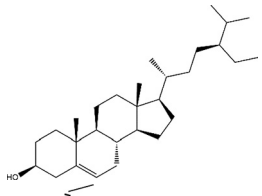
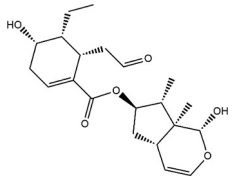
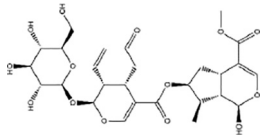
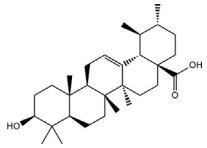
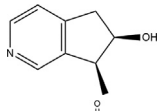
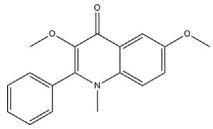
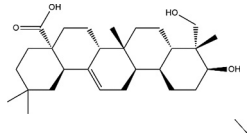
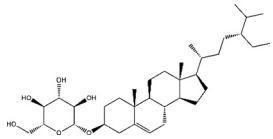
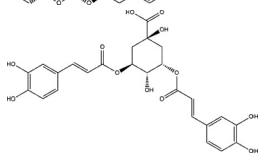
3. Results and discussions

3.1. Active compound identification

A total of 30 compounds of DR were retrieved from the TCMSP database (Supplementary material S1). By using the three in silico models, 7 ingredients of DR with favorable pharmacokinetic characteristics were screened out. In addition, though ursolic acid, sitogluside and venoterpine possess unsatisfactory pharmacokinetic properties, their therapeutic effects on OP have been proved by many studies. Here, to ensure the intact molecule database of DR, these 3 ingredients above were also included. Finally, 10 compounds were obtained as the potential active compounds (Table1).

These 10 potential compounds belong to different classes, including alkaloids, triterpenes, iridoids, steroid compounds, saponins, flavonoids and other compounds. Among of them, Beta-sitosterol (M02), belongs to steroid, possesses good pharmacokinetic characteristics (OB = 36.91%, DL = 0.75 and Caco-2 = 1.32), which promotes osteoblast proliferation by regulating the Wnt/β-catenin Signaling in vivo, thus exhibiting anti-osteoporotic effects [42,43,46]. As a flavonoid compound, gentisin (M01, OB = 64.06%, DL = 0.21 and Caco-

Table 1
The detail information of 10 candidate compounds.

Mol ID	Molecule name	Structure	Types	OB (%)	DL	Caco-2
M01	Gentisin		Flavonoids	64.06	0.21	0.60
M02	Beta-sitosterol		Steroids	36.91	0.75	1.32
M03	Sylvestroside III_qt		Iridoids	56.47	0.43	−0.34
M04	Sylvestroside III		Iridoids	48.02	0.53	−2.58
M05	Ursolic acid		Triterpenoid	16.77	0.75	0.67
M06	Venoterpine		Alkaloids	68.97	0.04	0.68
M07	Japonine		Alkaloids	44.11	0.25	1.25
M08	Cauloside a_qt		Saponins	43.32	0.81	−0.02
M09	Sitogluside		Others	20.63	0.62	−0.14
M10	(E,E)-3,5-Di-O-caffeoylquinic acid		Others	48.14	0.68	−1.31

2 = 0.6) exhibits good anti-inflammatory, antibacterial, diuretic functions, which could strengthen the kidney function to treat OP [47]. Sitogluside (M09, OB = 20.63%, DL = 0.62 and Caco-2 = −0.14) promotes osteoblast differentiation by regulating Wnt/β-catenin signaling pathway, thus enhancing bone formation [41,42]. Ursolic acid (M05, OB = 16.77%, DL = 0.75 and Caco-2 = 0.67) is a titerpenoid compound with various biological effects, such as anti-inflammatory, antibacterial and antioxidant effects [48]. Furthermore, it has been reported that ursolic acid possessed potent anti-osteoporotic effects by significantly suppressing RANKL-induced tartrate resistant acid phosphatase (TRAP) activity and multinucleate osteoclast formation [45]. It is noteworthy that RANKL signaling pathway is a part of OPG/RANKL/RANK, which is the most important signaling pathway in osteoimmunology [49]. These results indicated that DR may exhibit therapeutic effects on OP by the regulation of immune systems.

3.2. Target identification of DR for OP

For the 10 compounds, a total of 105 targets were obtained by the SysDT model (Supplementary material S2). Finally, 36 targets of 6 compounds associated with OP were identified by TTD, HIT and Genecards databases (4 compounds without targets). Finally, the detail

Table 2
The ligand-target interactions associated with anti-OP in DR.

MOL ID	Molecule name	Target name	Symbol
M01	Gentisin	Nitric oxide synthase, inducible	NOS2
M01	Gentisin	Aldose reductase	AKR1B1
M01	Gentisin	Estrogen receptor beta	ESR2
M01	Gentisin	Dipeptidyl peptidase IV	DPP4
M01	Gentisin	Mitogen-activated protein kinase 14	MAPK14
M01	Gentisin	Glycogen synthase kinase-3 beta	GSK3B
M01	Gentisin	cAMP-dependent protein kinase inhibitor alpha	PKIA
M02	Beta-sitosterol	Progesterone receptor	PGR
M02	Beta-sitosterol	Nuclear receptor coactivator 2	NCOA2
M02	Beta-sitosterol	Potassium voltage-gated channel subfamily H member 2	KCNH2
M02	Beta-sitosterol	Dopamine D1 receptor	DRD1
M02	Beta-sitosterol	Muscarinic acetylcholine receptor M3	CHRM3
M02	Beta-sitosterol	Alpha-1B adrenergic receptor	ADRA1B
M02	Beta-sitosterol	Beta-2 adrenergic receptor	ADRB2
M02	Beta-sitosterol	Apoptosis regulator Bcl-2	BCL2
M02	Beta-sitosterol	Apoptosis regulator BAX	BAX
M02	Beta-sitosterol	Caspase-9	CASP9
M02	Beta-sitosterol	Transcription factor AP-1	JUN
M02	Beta-sitosterol	Caspase-3	CASP3
M02	Beta-sitosterol	Transforming growth factor beta-1	TGFB1
M02	Beta-sitosterol	Mineralocorticoid receptor	NR3C2
M03	Sylvestroside III_qt	Dipeptidyl peptidase IV	DPP4
M03	Sylvestroside III_qt	Nuclear receptor coactivator 2	NCOA2
M05	Ursolic acid	Apoptosis regulator BAX	BAX
M05	Ursolic acid	Apoptosis regulator Bcl-2	BCL2
M05	Ursolic acid	Caspase-3	CASP3
M05	Ursolic acid	Caspase-9	CASP9
M05	Ursolic acid	Cathepsin B	CTSB
M05	Ursolic acid	Dual oxidase 2	DUOX2
M05	Ursolic acid	E-selectin	SELE
M05	Ursolic acid	Fatty acid synthase	FASN
M05	Ursolic acid	Intercellular adhesion molecule 1	ICAM1
M05	Ursolic acid	Interleukin-6	IL6
M05	Ursolic acid	Interstitial collagenase	MMP1
M05	Ursolic acid	Matrix metalloproteinase-9	MMP9
M05	Ursolic acid	Mitogen-activated protein kinase 8	MAPK8
M05	Ursolic acid	NF-kappa-B inhibitor alpha	NFKBIA
M05	Ursolic acid	Signal transducer and activator of transcription 3	STAT3
M05	Ursolic acid	Transcription factor AP-1	JUN
M05	Ursolic acid	Tumor necrosis factor	TNF
M05	Ursolic acid	Urokinase-type plasminogen activator	U-PA
M05	Ursolic acid	Vascular endothelial growth factor A	VEGFA
M06	Venoterpine	Alpha-1B adrenergic receptor	ADRA1B
M06	Venoterpine	Beta-2 adrenergic receptor	ADRB2
M09	Sitogluside	Progesterone receptor	PGR
M09	Sitogluside	Muscarinic acetylcholine receptor M3	CHRM3
M09	Sitogluside	Potassium voltage-gated channel subfamily H member 2	KCNH2
M09	Sitogluside	5-Hydroxytryptamine receptor 3A	HTR3A
M09	Sitogluside	Alpha-1B adrenergic receptor	ADRA1B
M09	Sitogluside	Beta-2 adrenergic receptor	ADRB2
M09	Sitogluside	Nuclear receptor coactivator 2	NCOA2

information of the interactions between compounds and targets was shown in Table 2.

These 36 targets mainly consist enzymes, nuclear receptors (NRs), transporters, g protein-coupled receptors (GPCRs), glycosaminoglycan binding proteins (GAGBPs), clusters of differentiation (CD) markers and transcription factors (Fig. 2A). Remarkably, enzymes account for the most proportion (38.89%, 14/36), including seven hydrolases/proteases, three transferases/kinases, three oxidoreductases (including one transporter), and one other enzymes (Fig. 2B). Among of hydrolases/proteases, apoptotic proteins Caspase-3 (CASP3) and Caspase-9 (CASP9) promotes the osteoblast apoptosis by activating the caspase-dependent apoptosis pathway [50,51].

GPCRs, a class of integral membrane proteins with seven transmembrane domains, account for the second largest proportion of the OP-targets (11.11%, 4/36). Among of them, b2-adrenoceptors (beta-2 adrenergic receptor, ADRB2) are able to regulate bone metabolism via decreasing bone resorption and increasing bone mineral density [52,53]. Besides, α1B-adrenoceptors (alpha-1B adrenergic receptor, ADRA1B) were regulated by noradrenaline, thus stimulating the proliferation of osteoblasts [53,54].

Transcription factors are proteins that bind to specific nucleotide sequences on the upstream of genes, which regulate gene expression by identifying and binding cis-acting elements in the gene promoter region [55,56]. Nuclear receptor coactivator 2 (NCOA2) is a transcription coactivator, which significantly decreases bone loss by the inhibition HIF-1α [57,58]. The other receptors are NRs, as ligand-activated transcription factors, representing 8.33% (3/36) of all protein targets. For example, progesterone receptors (PGR) bind to progesterone to reduce bone resorption and increase bone formation [59–62].

CD is a family of differentiated antigens that distributed on the surface of immune cells such as T cells, participating in the identification of adhesion and signal transduction in the immune response. CD18 is a CD marker expressed by BMSSCs, and its deficiency decreases BMD and increases trabecular bone space [53], indicating that DR may treat OP through the regulation of targets associated with the immune system.

3.3. GO enrichment analysis

GO enrichment results were visualized in Fig. 3. The OP-targets were involved in biological processes including Interleukin-6-mediated signaling pathway (*P*-value: 0.015662), response to estradiol (*P*-value: 0.020830), leukocyte tethering or rolling (*P*-value: 0.02083), osteoclast differentiation (*P*-value: 0.043763) and positive regulation of release of sequestered calcium ion into cytosol (*P*-value: 0.046279). Among of them, Interleukin-6-mediated signaling pathway and leukocyte tethering or rolling were associated with OP [63]. In addition, both Interleukin-6 and leukocyte are immune cells related to inflammations, in fact, inflammation is one of the important factor of OP, so the immune system maybe closely associated with bone metabolism [64–66]. These results indicated that the targets of DR were not only mapped into biological processes of the regulation of muscle contraction, the differentiation of osteoclasts, the absorption of collagen and the binding of hormones, but also associated with the immunology system. In summary, DR may possess the OP function by the regulation of both bone metabolism and immune systems.

3.4. C-T network

The C-T network was constructed based on the 6 candidate compounds and their 36 potential targets with 51 interactions (Fig. 4). The centralization and heterogeneity of the network were 0.424 and 1.424, respectively, indicating that some nodes are more concentrated in the network than others, that is, the compound-target space is biased toward certain compounds and targets.

The topological analysis demonstrated that compound with a high degree may exhibit pharmacological effects in OP. For example, M05 (ursolic acid, degree = 19) targeted on the highest number of target proteins, followed by M02 (beta-sitosterol, degree = 13), M01 (gentisin, degree = 7) and M09(sitogluside, degree = 7). Several studies have proved that these compounds play important roles in decreasing the bone loss, thereby treating OP [42–44,46,47]. In addition, the target with a high degree is considered as the key protein for treating OP. For instance, NCOA2 (degree = 3) significantly increased bone loss by inhibiting HIF-1α [57], and the inhibition of its expression can significantly improve the symptoms of OP [58]. HIF-1α protein inhibited pathological-activated osteoclasts in male OP patients [67]. ADRB2 is an important regulator in bone metabolism [52], and ADRA1B is suppressed by noradrenaline to modulate potassium

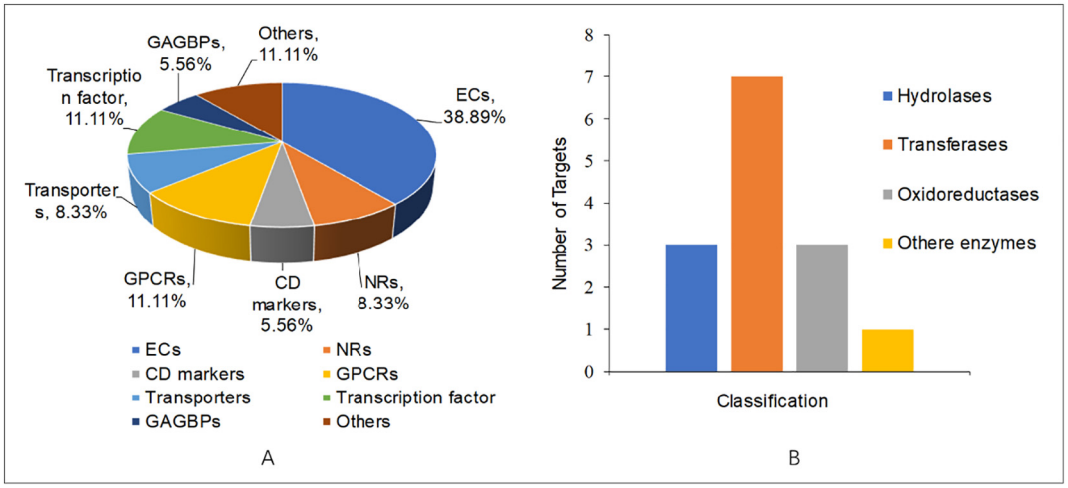


Fig. 2. (A) Distribution of drug targets according to their biochemical criteria. (B) Classification of targets in enzyme.

currents in the human osteoblast SaM-1 cell line [54]. PGR (degree = 2) is widely used as the therapeutic target in OP of postmenopausal women, which could bind to progesterone to reduce bone resorption and increase bone formation [59–62]. Actually, progesterone receptors are present on the surface of osteoblasts and they can directly target on PGR in osteoblasts [68,69].

Currently, the target proteins caspase-3 (CASP 3), caspase-9 (CASP 9), BCL2 and BAX have been reported to be involved in the apoptosis of osteoblasts [50,70]. IL-6 is a multi-functional cytokine, not only participate in immune regulation, inflammatory reaction and many other pathological processes, but also in the processes of the occurrence of OP. Besides, IL-6 can inhibit the osteoblast differentiation and stimulate the osteoclast proliferation, thus the inhibitors of IL-6 are effective for OP treatment [71]. TNF-α is produced by T lymphocytes, which plays an essential role in the inhibition of bone formation and mineralization by inhibiting alkaline phosphatase in osteoblasts [72,73]. Furthermore, TNF-α was able to increase the bone resorption of osteoclasts by inducing osteoclasts precursors to osteogenic differentiation [74,75]. Therefore, the inhibitors of TNF-α maybe possess the good therapeutic effects for OP. In summary, the C-T network analysis indicated that one compound might act on multiple targets and one target could be acted on multiple compounds, thus these compounds may combine to each other to enhance the therapeutic efficiency in treating OP at the holistic level.

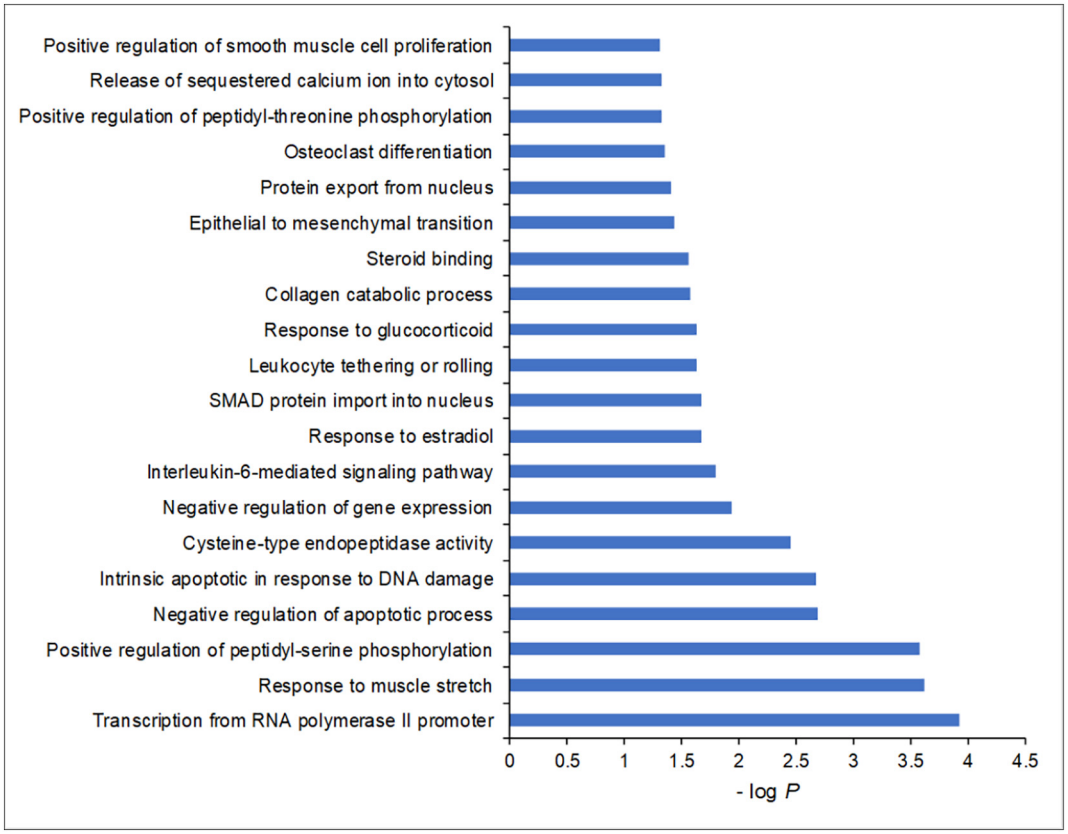


Fig. 3. GO enrichment analysis of anti-OP targets. The *x*-axis represents enrichment analysis ratings ($P < 0.05$) and the *y*-axis represents the top 20 biological processes.

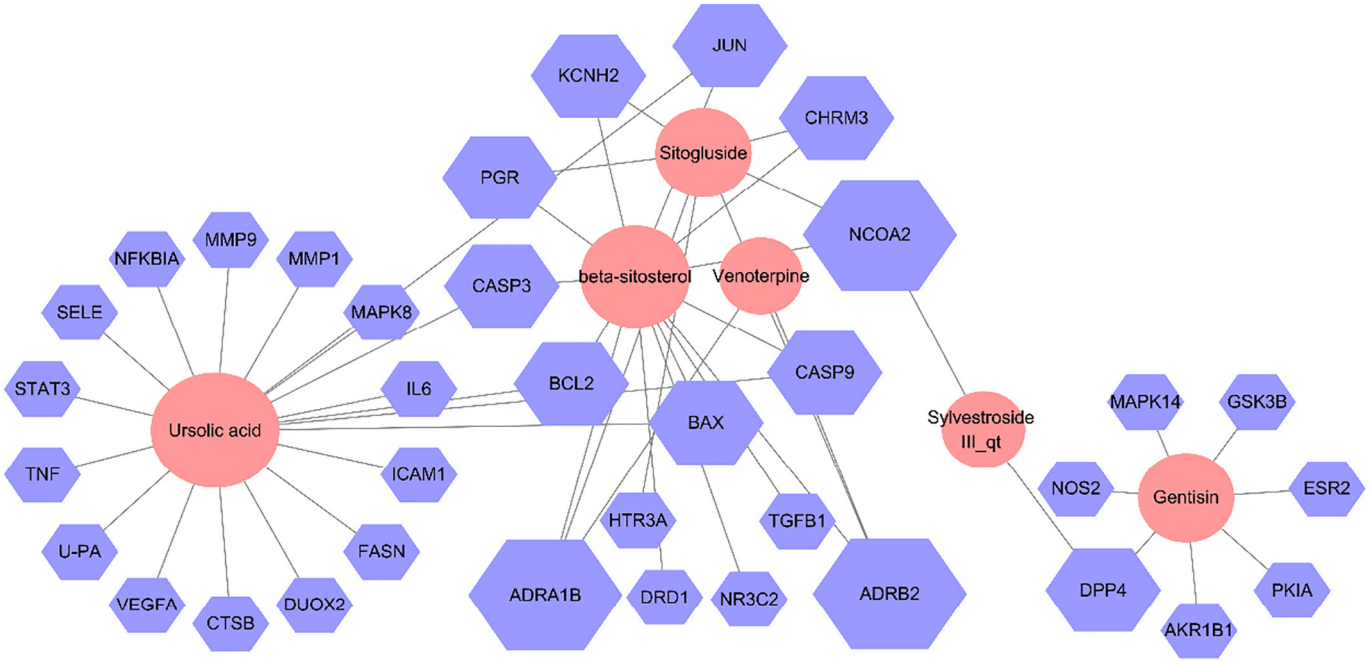


Fig. 4. The C-T network of DR for treating OP. The orange nodes represent active compounds, and the purple nodes represent drug targets. The edges represent the interaction between them, and the node size is proportional to their degree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

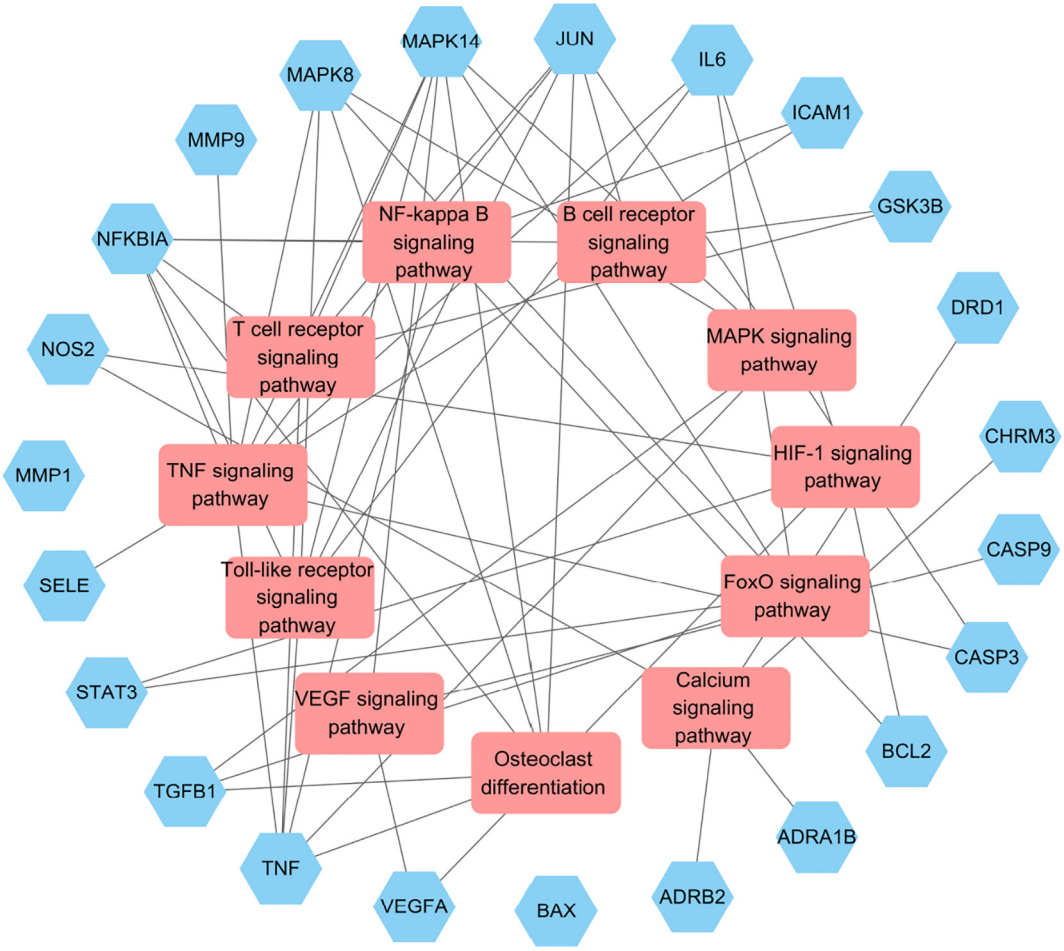


Fig. 5. T-P Network of DR for treating OP. The orange circle nodes represent pathways, and the blue isohexagon nodes represent the potential targets. The edges represent the interactions between them, and the node size is proportional to degree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

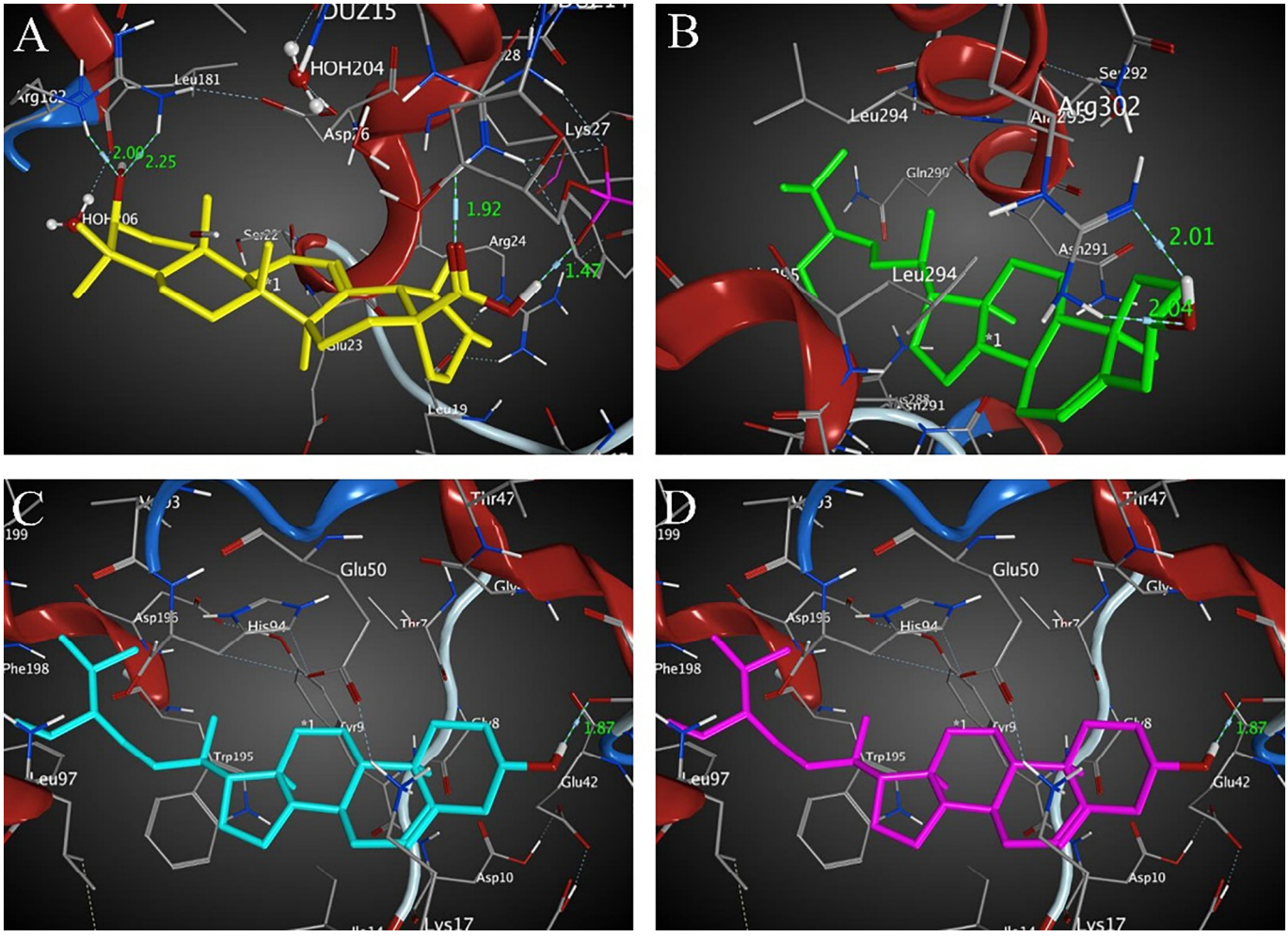


Fig. 6. Binding interactions of ursolic acid and beta-sitosterol with their ligands. A. ursolic acid_IL-6; B. beta-sitosterol_JUN; C. ursolic acid_BAX; D. beta-sitosterol_BCL-2.

3.5. T-P network analysis

In this section, the pathway enrichment analysis indicated that the predicted genes were significantly enriched in 11 pathways (Supplementary Material S3). The T-P network is composed of 34 nodes (11 pathways and 23 proteins) with 51 interactions (Fig. 5). In T-P network, the orange nodes represent the pathways, the blue nodes represent the compounds, and the edges represent the interactions between them. The centralization and heterogeneity of the network were 0.212 and 0.690, respectively. The major pathways were involved in inflammatory response and osteoclast differentiation, which are associated with OP, such as TNF signaling pathway (degree = 10), Toll-like receptor signaling pathway (degree = 6), Osteoclast differentiation signaling pathway (degree = 6), MAPK signaling pathway (degree = 6), NF-κb signaling pathway (degree = 4), Calcium signaling pathway (degree = 5) etc. Both osteoblasts and osteoclasts are derived from bone marrow primitive pluripotent stem cells, which play important roles in bone formation and bone resorption, respectively [76,77]. Besides, various systemic hormones and cytokines in the bone microenvironment act on the regulation of osteoblasts and osteoclasts to promote bone remodeling [78].

The balance between the bone formation and bone resorption could maintain bone remodeling and bone mass [79]. The targets of DR were involved in the signaling pathways associated with osteoblasts. For example, tumor necrosis factor (TNF) activated the NF-κb signaling pathway to regulate cell apoptosis and inflammation processes. In addition, TNF-α decreased phosphorylated Smad1 through the activation of NF-κb involved in BMP-2 signaling pathway, thus inhibiting osteoblast differentiation [74]. The mammalian family of mitogen-activated protein kinases (MAPKs) includes extracellular signal-regulated kinase (ERK), p38 and c-Jun NH 2-terminal kinase (JNK), which are the important factors in promoting the osteoblasts differentiation and mineralization [80,81]. Moreover, TNF-α inhibited osteoblast differentiation through MAPK signaling, suggesting that the inhibitors of TNF-α might be potential therapeutic targets of inflammation-induced bone loss [21,82]. In recent years, studies have demonstrated that Toll-like receptor 4 (TLR4) are contributed to the bone metabolism by regulating inflammatory reactions [83–87]. Moreover, TLR4 was also mapped in various signaling pathways which are closely related to osteoblasts [84,85,87]. Once the TLR4 related signaling pathway was activated, the expression of osteoblast markers, such as NF-κb ligand receptor activator (RANKL), Osteoprotegerin (OPG) and alkaline phosphatase (ALP) were suppressed, leading to the inhibition of osteoblast differentiation, proliferation and mineralization [50]. Furthermore, when the TLR4 pathway was inhibited, Wnt/β-catenin signaling pathway, TGF-β/BMP signaling pathway and Notch signaling pathway were activated to promote osteoblast differentiation [88,89]. These results suggest DR can regulate osteoblast proliferation, differentiation and mineralization by regulating MAPK, NF-κb and TLR4 signaling pathways.

On the other hand, these signaling pathways of the predicted targets are involved in the regulation of osteoclasts. The TNF-α increased bone

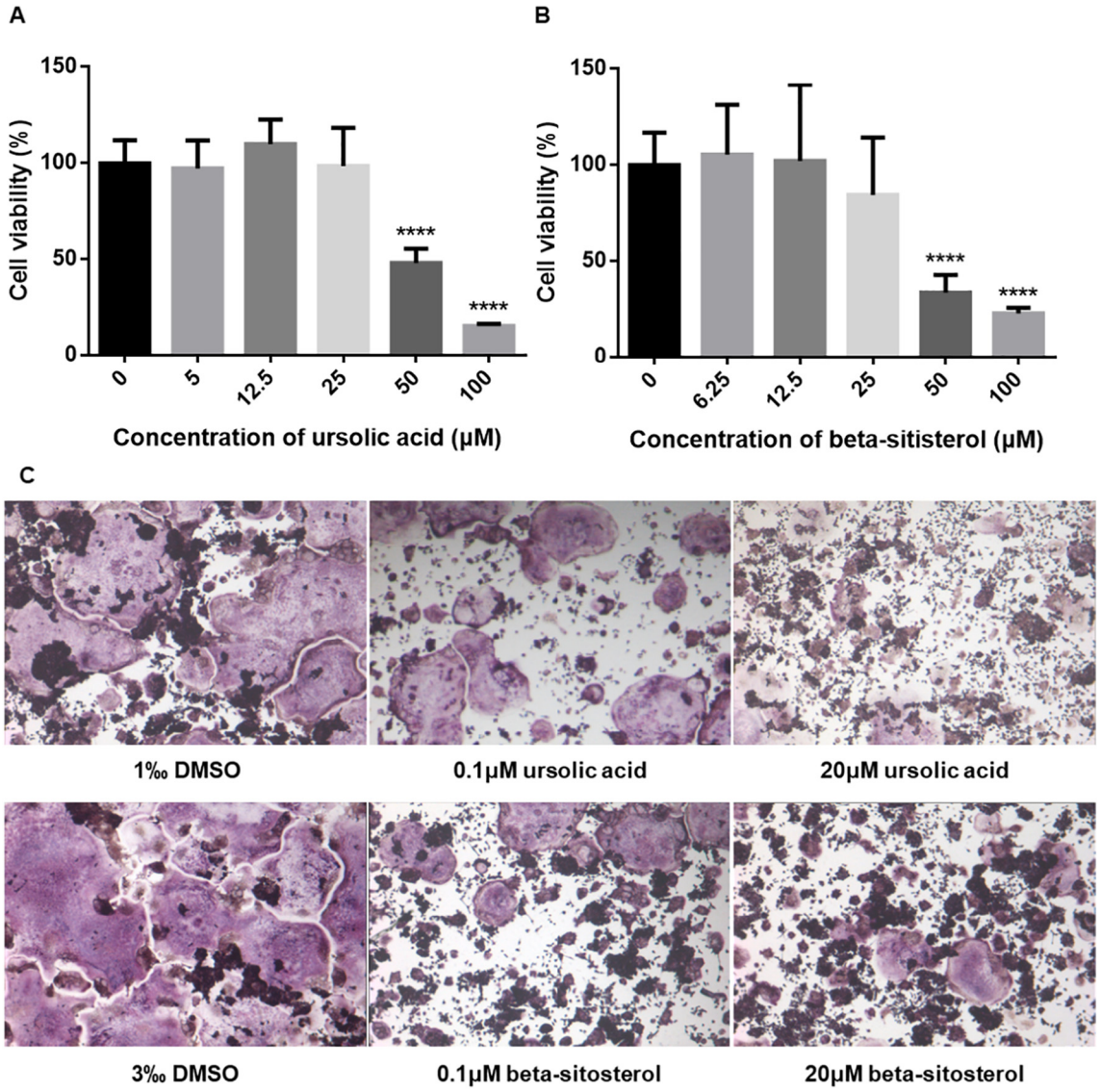


Fig. 7. The effect of active products on osteoclast differentiation of RAW 264.7 cells. A and B are the cell proliferation of ursolic acid and beta-sitosterol on osteoclast, respectively. C are TRAP staining results of osteoclast differentiation of RAW 264.7 cells. The concentrations of ursolic acid and beta-sitosterol are at 0.1 μM and 20 μM. ****: $P < 0.0001$.

loss not only by activating the osteoblast but also by connecting osteoclast regulation through Smad, MAPK, and NF-κb signaling pathways [82]. Besides, NF-κb signaling pathway combines with RANKL to enhance bone resorption by stimulating osteoclast differentiation. RANKL signaling pathway plays an important role in the osteoclast differentiation. Furthermore, OPG blocks the binding of NF-κb and RANKL, thereby inhibiting the osteoclast proliferation and differentiation [21,90]. Notably, OPG/RANKL/RANK system not only regulates the osteoclast differentiation, but also regulates immunity system [49]. Rupesh K et al. showed that activated T cells either directly or indirectly regulates bone systems through the secretion of various cytokines and factors [63]. The integrity of bone development is inextricably linked to the immune system, suggesting that the regulators of immune systems maybe a novel therapy for OP. Moreover, both MAPK signaling pathway and NF-κb signaling pathway are significant pathways associated with inflammation, which suggested that the anti-inflammation strategy could promote osteoblast differentiation and bone formation for treating OP [73]. In summary, DR may regulate bone balance between the resorption of osteoclasts and osteogenesis of osteoblasts to treat OP by the regulation of the immune system.

3.6. Molecular dynamics (MD) simulations

To validate the reliability of the above compound-target interactions and to further investigate the binding modes, we selected ursolic acid and beta-sitosterol with their corresponding targets which are approved and possess significant correlations with OP for MD analysis. Presently, 5 ns MD simulations for all the docked complexes were carried out to extract the kinetic conformational changes between the compounds and the targets, which occurred in aqueous solution. Moreover, the snapshots of the binding conformations of each complex with the key amino acids of the average structure at the last 1 ns of the MD simulations are depicted in Fig. 6. Obviously, ursolic acid and beta-sitosterol are located within the binding cavity of their corresponding targets. Ursolic acid binding with IL-6 and BAX via hydrogen bonds interaction, which help the stabilization of these compounds at the binding site. In addition, beta-sitosterol binding with BCL-2 and JUN via hydrogen bond interactions.

3.7. The effects of active compounds on osteoclast differentiation

In this section, the cell proliferation of ursolic acid and beta-sitosterol were shown in Fig. 7 A and B, respectively. The results indicated that ursolic acid and beta-sitosterol had no side effects on RAW 264.7 when the concentrations of ursolic acid were < 25 μM (Fig. 7 A, B). The TRAP staining results showed that ursolic acid inhibited the osteoclast differentiation only at the concentration of 20 μM, and beta-sitosterol with the concentration of 0.1 μM and 20 μM (Fig. 7C), which were consistent with the predicted results.

4. Conclusions

Systems pharmacology was performed to dissect the molecular mechanisms of DR for the treatment of OP. A total of 6 bioactive compounds and their related 36 targets were identified in DR, and these targets were mostly involved in the regulation of bone balance between the bone formation of osteoblasts and the bone absorption of osteoclasts. The network analysis indicated that DR may regulate immune signaling pathways for the management of OP. The in vitro results indicated that the compounds ursolic acid and beta-sitosterol inhibited the osteoclast differentiation, which was consistent with the predicted results. In summary, the action mechanism of DR was dissected from the molecular and systematic level, which lays a foundation for the novel effective anti-OP drug development.

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Author contributions and email address

Airong Qian (E-mail: qianair@nwpu.edu.cn) and Wenjuan Zhang (E-mail: wenjuan_zhang2017@nwpu.edu.cn) designed the paper and supervised the research. Wenjuan Zhang (E-mail: wenjuan_zhang2017@nwpu.edu.cn) performed the research and wrote the paper. Kaiyue Xue (E-mail: Kaiyue kaiyuexue@mail.nwpu.edu.cn) took the cell experiments and draw figures. Yongguang Gao (E-mail: gaoyongguang@nwpu.edu.cn) revised the manuscript. Ying Huai (E-mail: huai_@mail.nwpu.edu.cn), Wei Wang (E-mail: wangwei0206@mail.nwpu.edu.cn), Kai Dang (E-mail: dangkai@nwpu.edu.cn), Shanfeng Jiang (E-mail: jiang2017@nwpu.edu.cn) and Zhiping Miao (E-mail: miaozp@nwpu.edu.cn) prepared tables and figures. All authors reviewed the manuscript.

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Declaration of competing interest

We declare that there is no conflict of interests.

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