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Article

Research on Molecular Mechanism of Fructus Ligustri Lucidi against Osteoporosis based on Network Pharmacology

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TCMSP platform of systematic pharmacology of traditional Chinese medicine

This study aimed to investigate the molecular mechanism of Fructus Ligustri Lucidi (NZZ, Chinese abbreviation) against osteoporosis (OP) by means of network pharmacology.ChemDraw Professional 15.1 software and Molinspiration Smiles database were used to draw the chemical formulas of the components. The active ingredients and related target proteins of NZZ were searched in platform of systematic pharmacology of traditional Chinese medicine database, Drugbank, Therapeutic Target Database, SymMap and other databases. Gene Ontology(GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were carried out on the selected target through Enrichr and KEGG Automatic Annotation databases, and their mechanism was studied. A total of 29 compounds and 140 corresponding targets, including 14 key targets and 14 protein factors in protein-protein interaction core network were obtained. The key targets were tumor necrosis factor(TNF), interleukin(IL)-6R and sestrogen receptor alpha. The number of GO items was 466 ($P<0.05$), including 399 items of biological process (BP), 54 items of cell composition (MF) and 13 items of molecular function (CC). KEGG pathway enrichment screened 85 signaling pathways ($P<0.05$), including the IL-17 signaling pathway, TNF signaling pathway, advanced glycation end products and their receptors signaling pathway and cAMP signaling pathway. The active ingredients of NZZ. exert their anti-OP effects through multi-components, multi-targets and multi-pathways, which can provide new evidence for further study of their anti-OP mechanism.

Keywords: Fructus Ligustri Lucidi. Traditional Chinese medicine. Network pharmacology. Osteoporosis. Target.

INTRODUCTION

Osteoporosis(OP) is a metabolic osteopathy characterized by abnormal bone mass and bone tissue microstructures. Its mechanism is extremely complex and involves many unrelated pathogeneses. OP occurs in different genders and at any age, but mostly in postmenopausal women and middle-aged and elderly men. The main features of OP are pain and easy to fracture skeleton. OP can be divided into primary and secondary types and affects more than 200 million people in the world, thus greatly increasing the proportion of human abnormal death. (Kastner *et al*., 2014)

Traditional Chinese medicine (TCM), includes the medicines originating in China. TCM differs from Western medicine, and its components are mixed. A TCM often has numerous components, that can act on many disease targets. The multi-component and multi-target functions of TCM make it important in the treatment of difficult and complicated diseases.

Fructus Ligustri Lucidi(NZZ) belongs to the fruit of NZZ, which contains desirable anti-OP active ingredients, which is popular among people. In addition, NZZ has anti-inflammatory, anti-tumor, hypolipidemic, hypoglycemic and immunomodulatory active ingredients (Ngo *et al*.,

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2017). The research on the chemical constituents and pharmacological effects of NZZ has been increasing in China, which has gradually attracting the interest of clinical researchers toward the anti-OP mechanism of NZZ(Gan *et al*., 2019; Liu *et al*., 2015). However, although the research on anti-OP targets is ongoing, a target or drug that can cure anti-OP completely remains unavailable. The number of drugs used to treat OP in clinic is limited, and their adverse reactions are serious. Therefore, we need to further study the pathogenesis and therapeutic of OP.

To understand the anti-OP mechanism of NZZ, based on systematic pharmacology and network pharmacology, we obtained the possible anti-OP targets and related genes of NZZ, determined the relationship between components and targets through target component interaction network, and identified the core targets through a protein protein interaction (PPI) network. Finally, the paths and potential mechanisms of NZZ were analyzed by pathology and physiology.

MATERIAL AND METHODS

Relevant Data Collection

All known active ingredients of NZZ were obtained on the database and analysis Platform of Systematic Pharmacology of Traditional Chinese Medicine (http://lsp.nwu.edu.cn/, TCMSP). In accordance with the requirement of TCMSP for Chinese medicines with high utilization value, eligible compounds were screened from the above active ingredients. ChemDraw Professional 15.1 software was used to draw the chemical formulas of the selected components and save them in smiles format. The files saved in smiles format were imported into Molinspiration Smiles (https://www.molinspiration.com) database and the qualified compounds were screened following Linpinski's five-fold rule. Disease information in the TCMSP database, which can be queried and downloaded originated from the Therapeutic Target Database (TTD) and Pharmacogenomics Knowledgebase (PharmGKB). PharmGKB uniquely provided pharmacokinetic information for each compound. Users can select compounds with good drug-like and ADME (absorption, distribution, metabolism, and excretion) characteristics for further research.

Target Prediction and Construction of the Component Target Network

The Swiss Target Prediction (http://www.swisstargetprediction.ch) database was used to predict the active targets of the above active ingredients. We used Cytoscape 3.6.1 software to construct protein-protein (PPI) and component-target interaction networks. Target protein molecules are represented by "nodes" and the interrelationships by "edges". With the excellent visual interface of Cytoscape 3.6.1, which is the most trustworthy software at present, the interaction between components and targets can be displayed.

Retrieval of OP Targets Used in Clinical Therapy

With "OP" as the key word, we used TTD (http://bid.nus.edu.sg/BIDD-Databases /TTD/TTD.asp) database and DrugBank (http://www.drugbank.ca) database to search and screen known OP-related targets, and found their corresponding gene names in UniProt database.

Gene Ontology (GO) Enrichment Analysis

The obtained targets of compounds into were imported into the Enrichr gene enrichment database. (http://amp.pharm.mssm.edu/Enrichr/, Enrichr). We obtained several GO items ($P<0.05$), including biological process (BP), cellular component (CC), and molecular function (MF).

Using Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

Using KEGG automatic annotation database (https://www.kegg.jp/blastkoala/) was used to analyze the obtained targets and obtain OP-related signaling pathways ($P<0.05$).

Molecular Docking Technologies

The ligands and water molecules in the target proteins were removed by Pymol software, and the active components of NZZ were transformed into mol2 format by OpenBabel software. Swissdock(http://www.swissdock.ch/) was used to connect the structure files of the target proteins and small molecules.

RESULTS AND DISCUSSION

Active Constituents of NZZ

A total of 119 active ingredients of NZZ were obtained from TCMSP database,. In accordance with the requirement of TCMSP for Chinese medicines with high utilization value, 34 active ingredients were screened out of 119 active ingredients based on the principle of negative logarithmic value of lipid water partition coefficient (-lg(P)>1.3013) and class DL (>0.18). Using Molinspiration Smiles database and Linpinski's five-fold rule, 29 components including salidroside, daidzein, luteolin, quercetin, kaempferol and hydroxy tyrosine were obtained.

TABLE I- Linpinski Five Screening Components of NZZ

Ingredients	-LogP≤5	M mass 160-480	Hbond acceptor≤10	Hbond donor ≤5
salidroside	FIT	FIT	FIT	FIT
daidzein	FIT	FIT	FIT	FIT
Lucidumoside D_qt	FIT	FIT	FIT	FIT
luteolin	FIT	FIT	FIT	FIT
apigenin	FIT	FIT	FIT	FIT
quercetin	FIT	FIT	FIT	FIT
kaempferol	FIT	FIT	FIT	FIT
Vomifoliol	FIT	FIT	FIT	FIT
eugenol	FIT	FIT	FIT	FIT
(-)-nopinene	FIT	FIT	FIT	FIT
hydroxytyrosol	FIT	FIT	FIT	FIT
Sinapyl alcohol	FIT	FIT	FIT	FIT
L-Bornyl acetate	FIT	FIT	FIT	FIT
(R)-linalool	FIT	FIT	FIT	FIT
caffeic acid	FIT	FIT	FIT	FIT
L-Limonen	FIT	FIT	FIT	FIT
(-)-Borneol	FIT	FIT	FIT	FIT
Methylcinnamate	FIT	FIT	FIT	FIT

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TABLE I- Linpinski Five Screening Components of NZZ

Ingredients	-LogP≤5	M mass 160-480	Hbond acceptor≤10	Hbond donor ≤5
Coniferol	FIT	FIT	FIT	FIT
(-)-Olivir	FIT	FIT	FIT	FIT
Damascenone	FIT	FIT	FIT	FIT
alpha-L-Rhamnose	FIT	FIT	FIT	FIT
taxifolin	FIT	FIT	FIT	FIT
ketologanin_qt	FIT	FIT	FIT	FIT
Oleoside dimethyl ester_qt	FIT	FIT	FIT	FIT
alpha-Methyl-d-galactoside	FIT	FIT	FIT	FIT
eriodictyol	FIT	FIT	FIT	FIT
10-Hydroxyoleoside dimethyl ester_qt	FIT	FIT	FIT	FIT
Dibutyl phthalate	FIT	FIT	FIT	FIT

Target Prediction and Network Graph Analysis

Using the Swiss Target Prediction database, 14 OP-related targets were predicted, including estrogenreceptor alpha (ESR1), prostaglandin G/H synthase 2 (PTGS2), and beta-2 adrenergic receptor (ADRB2). Using Cytoscape to construct the PPI network and component target interaction networks, we observed that 140 PPI-related targets were mapped after 14 protein factors have interacted. Based on the PPI enrichment P value: <1.0e-16, three of the most interacting nodes were transcription factor AP-1 (JUN), mitogen-activated protein kinase 14 (MAPK14) and tumor necrosis factor(TNF). TNF receptor-related factor 6 MAPK8 and ESR1 were the most potent factors. The component-target interaction network showed that 29 OP-related active ingredients interacted with 14 gene targets. On the basis of the effect, the targets was arranged from the strongest to the weakest as follows, in order of PTGS2, ADRB2, TNF, JUN, ESR1, arachidonate 5-lipoxygenase (ALOX5), interleukin receptor 6 (IL6R), and cytochrome P450 1A2 (CYP1A2).

TABLE II- Predicted targets for NZZ

Protein name	Uniprot ID	Gene symbol
Prostaglandin G/H synthase 2	P35354	PTGS2
Estrogen receptorI	P03372	ESR1
Beta-2 adrenergic receptor	P07550	ADRB2
Mitogen-activated protein kinase 14	Q16539	MAPK14
Arachidonate 5-lipoxygenase	P09917	ALOX5
CGMP-inhibited 3',5'-cyclic phosphodiesterase A	Q14432	PDE3A
C-C motif chemokine 2	P13500	CCL2
Cytochrome P450 1A2	P05177	CYP1A2
Collagen alpha-1(I) chain	P02452	COL1A1
Interleukin-6 receptor	P08887	IL6R
Stromelysin-1	P08254	MMP3
tumor necrosis factor	P01375	TNF
Transcription factor AP-1	P05412	JUN
Interstitial collagenase	P03956	MMP1

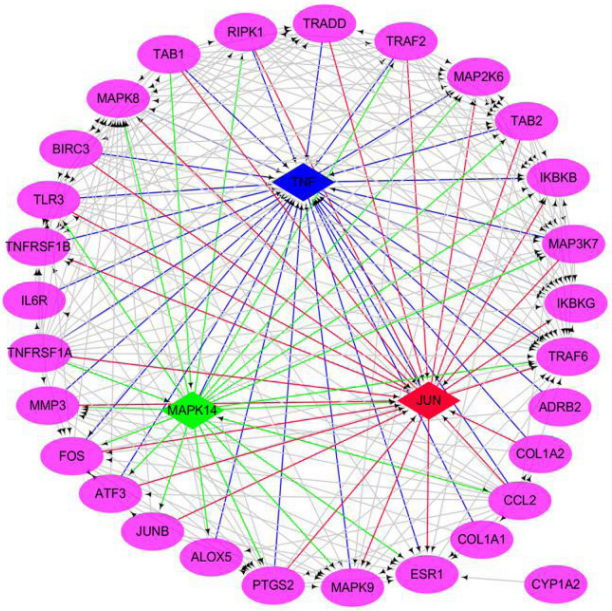

FIGURE 1-Protein-Protein Interaction Network (PPI)

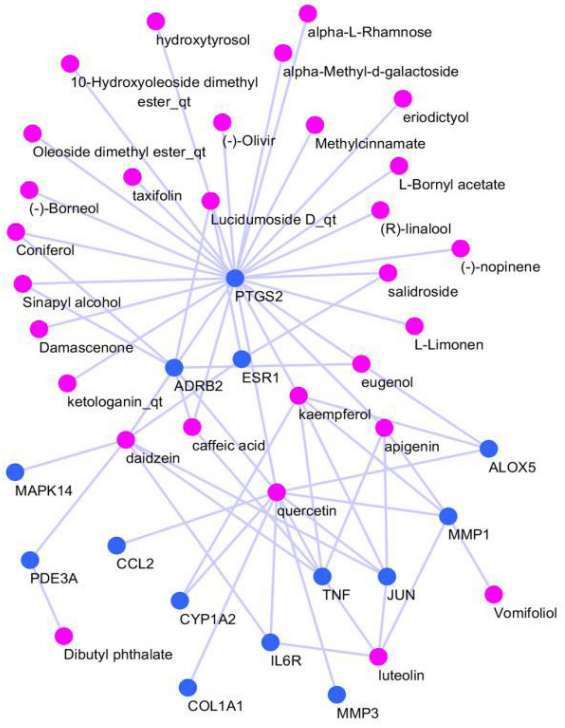

FIGURE 2-Components-Targets Interaction Network

Anti-OP Targets Used Clinically

With "osteoporosis" as a key word, we used TTD and DrugBank database to search and screen the known OP-related targets, and find the corresponding gene names in UniProt database. We have retrieved 34 clinical targets for OP or related symptoms. These targets included the nuclear factor kappa B receptor activating factor ligand (RANKL), parathyroid hormone receptor and WNTL signaling pathway.

Intersection of NZZ Action and Clinical Targets

By comparing the targets of the active components of NZZ with the current anti-OP targets in clinic, we observed that the targets of direct intersection (the same) were beta-2 adrenergic receptor (ADRB2) and ESR1; the targets of intersection were PTGS2, TNF, IL6R and cGMP inhibition. The non-intersecting targets were MAPK14, ALOX5, JUN, interstitial collagenase (matrix metalloproteinase (MMP) 1), estrogen-1 (MMP3), C-C chemokine 2, collagen alpha-1 (I) chain and CYP1A2.

SUPPLEMENTAL TABLE I -Anti-OP targets used clinically. Red indicates intersection

Gene Symbols of Clinical Targets		
CASR	HA	CTSK
PTGER2*	WNTL	LTB4R
RANKL	PTK	STS
PGR	ITGB5	CNR2
SRC	ADR*	ESR1*
VDR	DKK1	ODFR
ALPL	SOST	LIF
PTPRS	IL3R*	PDE7A*
PTH1R	IL5A3B*	ITGR
FDPS	HSD17B	ACV
CALCR	RUNX2	
TNFSF11*	PTK2	

Note: *indicates intersection

Results of GO Enrichment Analysis

Using Enrichr database for GO enrichment analysis, we obtained 466 GO entries (such as type I-II diabetes mellitus.P<0.05), of which 399 belong to BP, including cytokine-mediated signaling pathway, positive regulation of cell differentiation, positive regulation of acute inflammatory response, intracellular ESR signaling pathway, positive regulation of MAPK cascade, positive regulation of protein serine/threonine kinase activity, and positive regulation of protein serine/threonine kinase activity. A total of 21 key processes were observed in the regulation of vitamin D biosynthesis process; The MF entries included 12 key functions, such as oxidoreductase activity, metal endopeptidase activity, heme binding, DNA binding in transcription regulatory region, adrenergic receptor activity, and CC comprised 13 items, including 11 key components such as cytoplasmic cavity, fibrous gel protein enriched granule, nuclear chromatin and endoplasmic reticulum cavity, and so on.

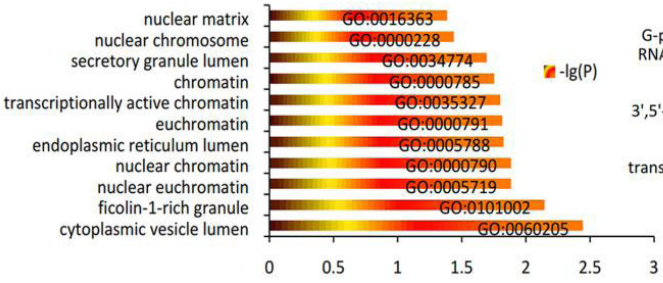

FIGURE 3-Cellular component

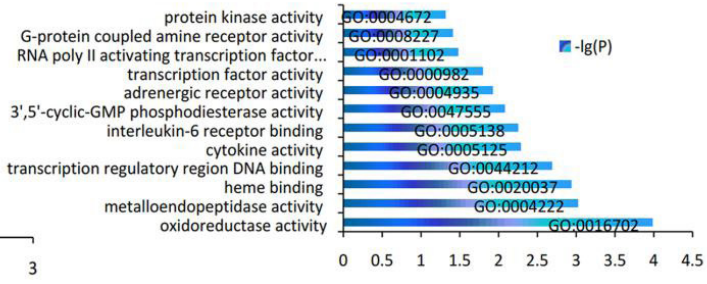

FIGURE 4-Molecular functions

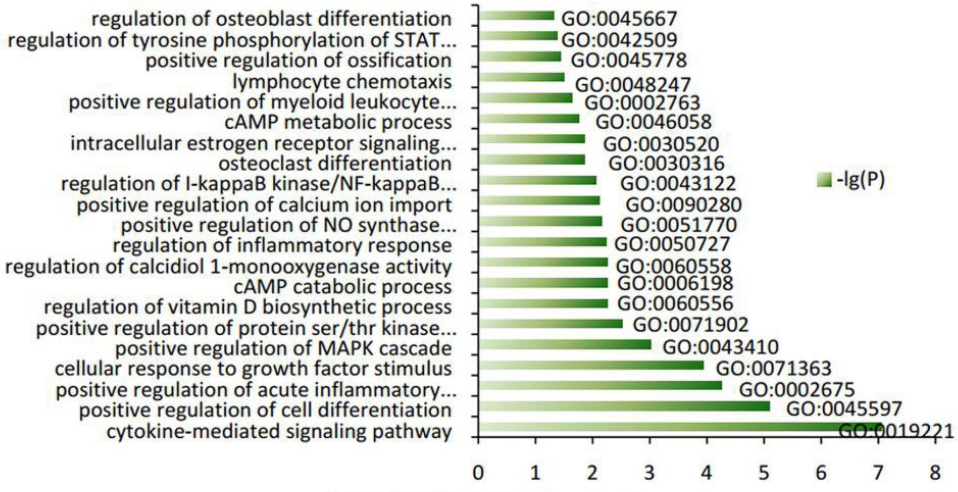

FIGURE 5-Biological process

Results of KEGG Pathwaây Analysis

We used KEGG database to obtain 85 OP-related signaling pathways (P<0.05), including IL17 signaling pathway, TNF signaling pathway, rheumatoid arthritis pathway, advanced glycation end products and their receptors (AGE-RAGE) signaling pathway of diabetic complications, osteoclast differentiation, estrogen signaling pathway, cGMP-PKG signaling pathway, asthma, and so on.

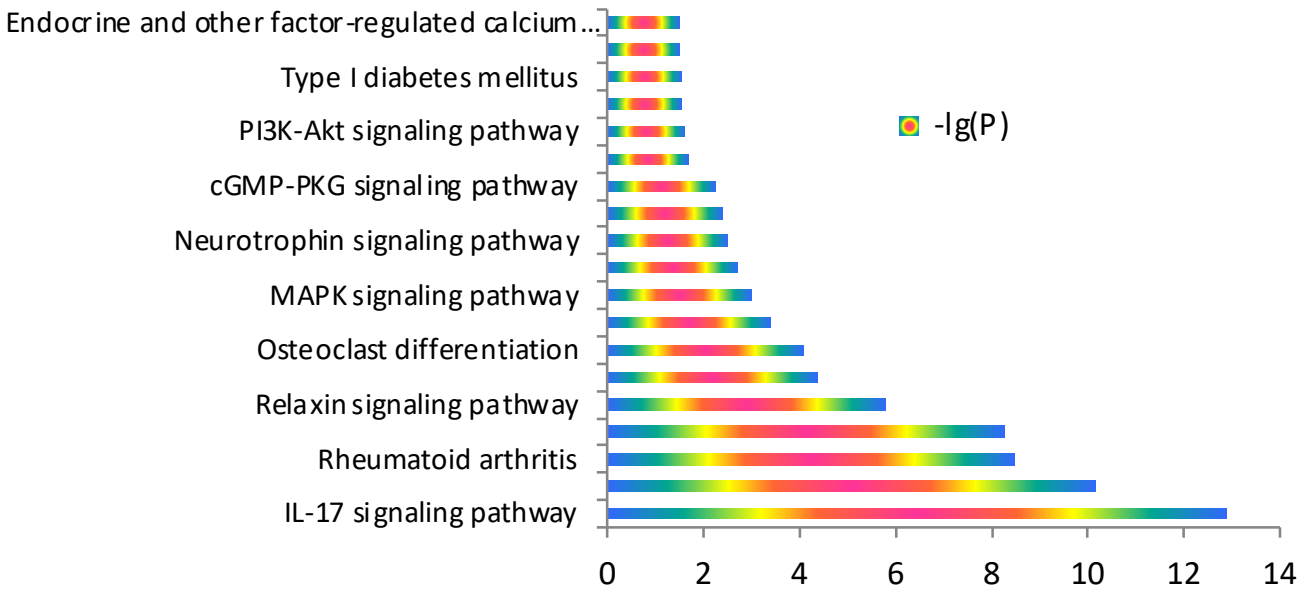

FIGURE 6 - KEGG pathway

SUPPLEMENTAL TABLE II The glossary in this list

Abbreviation	Full form
NZZ	fructus ligustri lucidi
OP	osteoporosis
GO	gene ontology
KEGG	kyoto encyclopedia of genesand genomes
PPI	protein-protein interaction
TNF	tumor necrosis factor
IL-6R	human interleukin 6 receptor
ESR1	estrogen receptor 1
BP	biological process
MF	cell composition
CC	molecular function
IL-17	interleukin 17
AGE-RAGE	signaling pathway in diabetic complications
CAMP	cyclic adenosine monophosphate
TCM	Traditional Chinese medicine
ADME	absorption, distribution, metabolism, excretion
PTGS2	prostaglandin G/H synthase 2

SUPPLEMENTAL TABLE II The glossary in this list

Abbreviation	Full form
ADRB2	beta-2 adrenergic receptor
JUN	Transcription factor AP-1
MAPK14	mitogen-activated protein kinase 14
TRAF6	Necrosis factor receptor-related factor 6
MAPK8	mitogen-activated protein kinase 8
ALOX5	Arachidonate 5-lipoxygenase
CYP1A2	Cytochrome P450 1A2
RANKL	nuclear factor kappa B receptor activating factor ligand
PTH1R	parathyroid hormone receptor
PDE3A	CGMP-inhibited 3',5'-cyclic phosphodiesterase A
MAPK14	Mitogen-activated protein kinase 14
MMP1	Interstitial collagenase
MMP3	Stromelysin-1
CCL2	C-C motif chemokine 2
COL1A1	Cytochrome P450 1A2
MAPK	Mitogen-activated protein kinase
NF-κB	nuclearfactorκB

Results of molecular docking technologies

SUPPLEMENTAL TABLE III The results of molecular docking technologies in this list

Protein name	Gene symbol	Ingredients	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
Prostaglandin G/H synthase 2	PTGS2	salidroside	-3432.26	-7.6
Estrogen receptorI	ESR1	salidroside	-1197.88	-7.93
Beta-2 adrenergic receptor	ADRB2	salidroside	-3059.6	-7.42
Interleukin-6 receptor	IL6R	salidroside	-3894.11	-8.44
Transcription factor AP-1	JUN	salidroside	-1701.97	-6.87
Interstitial collagenase	MMP1	salidroside	-2743.57	-6.93
Prostaglandin G/H synthase 2	PTGS2	daidzein	-3481.33	-7.4
Estrogen receptorI	ESR1	daidzein	-1250.92	-7.95
Beta-2 adrenergic receptor	ADRB2	daidzein	-3108.18	-7.09
Interleukin-6 receptor	IL6R	daidzein	-3943.99	-7.54
Transcription factor AP-1	JUN	daidzein	-1750.56	-6.49
Interstitial collagenase	MMP1	daidzein	-2793.58	-6.94
Prostaglandin G/H synthase 2	PTGS2	luteolin	-3500.18	-7.62
Estrogen receptorI	ESR1	luteolin	-1264.09	-7.61
Beta-2 adrenergic receptor	ADRB2	luteolin	-3126.81	-7.25
Interleukin-6 receptor	IL6R	luteolin	-3960.2	-7.5
Transcription factor AP-1	JUN	luteolin	-1768.85	-6.42
Interstitial collagenase	MMP1	luteolin	-2811.26	-6.88
Prostaglandin G/H synthase 2	PTGS2	apigenin	-3503.43	-7.56
Estrogen receptorI	ESR1	apigenin	-1268.98	-7.76
Beta-2 adrenergic receptor	ADRB2	apigenin	-3129.12	-6.9
Interleukin-6 receptor	IL6R	apigenin	-3963.19	-7.47
Transcription factor AP-1	JUN	apigenin	-1770.93	-6.22
Interstitial collagenase	MMP1	apigenin	-2813.24	-6.76
Prostaglandin G/H synthase 2	PTGS2	quercetin	-3478.42	-7.37
Estrogen receptorI	ESR1	quercetin	-1243.47	-7.86
Beta-2 adrenergic receptor	ADRB2	quercetin	-3106.38	-7.04
Interleukin-6 receptor	IL6R	quercetin	-3939.44	-7.63
Transcription factor AP-1	JUN	quercetin	-1749.95	-6.71
Interstitial collagenase	MMP1	quercetin	-2792.39	-7

(continues on the next page...)

SUPPLEMENTAL TABLE III The results of molecular docking technologies in this list

Protein name	Gene symbol	Ingredients	FullFitness (kcal/mol)	Estimated ΔG (kcal/mol)
Prostaglandin G/H synthase 2	PTGS2	kaempferol	-3478.54	-6.89
Estrogen receptorI	ESR1	kaempferol	-1245.6	-7.67
Beta-2 adrenergic receptor	ADRB2	kaempferol	-3110.41	-7.21
Interleukin-6 receptor	IL6R	kaempferol	-3940.87	-7.16
Transcription factor AP-1	JUN	kaempferol	-1752.79	-6.28
Interstitial collagenase	MMP1	kaempferol	-2793.55	-6.85
Prostaglandin G/H synthase 2	PTGS2	eugenol	-3479.99	-6.83
Estrogen receptorI	ESR1	eugenol	-1242.91	-6.89
Beta-2 adrenergic receptor	ADRB2	eugenol	-3105.53	-6.31
Interleukin-6 receptor	IL6R	eugenol	-3940.66	-6.92
Transcription factor AP-1	JUN	eugenol	-1749.34	-5.93
Interstitial collagenase	MMP1	eugenol	-2793.55	-6.4
Prostaglandin G/H synthase 2	PTGS2	Sinapyl alcohol	-3482.84	-7.14
Estrogen receptorI	ESR1	Sinapyl alcohol	-1246.77	-7.18
Beta-2 adrenergic receptor	ADRB2	Sinapyl alcohol	-3110.18	-6.99
Interleukin-6 receptor	IL6R	Sinapyl alcohol	-3942.17	-6.82
Transcription factor AP-1	JUN	Sinapyl alcohol	-1751.06	-6.04
Interstitial collagenase	MMP1	Sinapyl alcohol	-2800.4	-7.12
Prostaglandin G/H synthase 2	PTGS2	caffeic acid	-3515.25	-6.49
Estrogen receptorI	ESR1	caffeic acid	-1282.64	-7.04
Beta-2 adrenergic receptor	ADRB2	caffeic acid	-3145.87	-6.96
Interleukin-6 receptor	IL6R	caffeic acid	-3978.27	-7.11
Transcription factor AP-1	JUN	caffeic acid	-1789.02	-6.31
Interstitial collagenase	MMP1	caffeic acid	-2833.28	-6.68
Prostaglandin G/H synthase 2	PTGS2	Coniferol	-3494.08	-7.1
Estrogen receptorI	ESR1	Coniferol	-1262.42	-7.39
Beta-2 adrenergic receptor	ADRB2	Coniferol	-3120.64	-7.03
Interleukin-6 receptor	IL6R	Coniferol	-3954.36	-7.11
Transcription factor AP-1	JUN	Coniferol	-1765.14	-6.02
Interstitial collagenase	MMP1	Coniferol	-2811.23	-6.75

DISCUSSION

Nearly 20 years of history has passed since human beings entered the post-genomic era. Detection of gene sequences is no longer an important direction of human research on diseases, but focus should shift to the study of gene function, gene drugs, and bioinformatics. The mechanism of NZZ in the treatment of OP based on network pharmacology is the core of bioinformatics research in the post-genomic era. (Chen *et al*., 2015; Ouni *et al*., 2019)

NZZ has a notably distinct anti-OP effect, which is mainly reflected in its active ingredients, including luciferin D_qt, salidroside, quercetin, daidzein, sinapyl alcohol, caffeic acid, coniferol and eugenol, which can act on ADRB2 and ESR1 receptors(Chen *et al*., 2017). Furthermore, ESRs can be expressed in all cells related to bone resorption and bone formation, especially when ESR1 receptor is inhibited, these receptors can directly reduce the number of osteoclasts. (Bukhari *et al*., 2019; Liu *et al*., 2015; Song *et al*., 2015)

Lei's research showed that patients with endocrine and metabolic diseases (diabetes, and hyperthyroidism, etc.), rheumatoid diseases (rheumatoid arthritis, etc.), hematological diseases (anemia, leukemia, etc), and kidney diseases (renal failure, chronic nephritis, etc) are all at risk of OP. However, it is interesting to note that NZZ may also have some therapeutic effects on secondary OP (Lei MM 2018).

On the basis of PPI network and components-targets networks, we performed GO enrichment analysis, and compared the results with the clinical anti-OP targets. The results showed that NZZ can act on OP and related diseases through ESR1, ADRB2 and PTGS2 targets. Moreover, it is gratifying that these targets are mutual and directly correspond to salidroside and daidzein in NZZ.

Estrogen is an important factor to improving bone density and prevent bone loss after menopause. ESR (estrogen receptor) is found on the surface of human osteoblasts and osteoclasts, which are involved in estrogen signal transduction pathway. Given the considerable number of ADRB2 receptors in bone tissue, nuclear factor-κB signaling pathway can be used to treat OP by inhibiting it through the reduction of its expression level. PTGS2_(or COX-2), is abundant in inflammatory cells and can reduce inflammation by inhibiting the IL-17 signaling pathway. Coincidentally, the above conclusions are consistent with the results of KEGG pathway analysis.

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