Study on the mechanism of Guilu Erxian gum in the treatment of osteoporosis based on network pharmacology and cell experiment

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Abstract

The aim of this study is to investigate how Guilu Erxian gum treats osteoporosis by using network pharmacology and related experiments. Methods: The TCMSP database, ETCM database, Chemical Specialties database, and DrugBank database were used to retrieve the potential active ingredients and corresponding targets of Guilu Erxian gum. Therapeutic targets associated with osteoporosis were obtained from the OMIM and GeneCards databases. The network was analyzed using Cytoscape 3.8.0 software to determine topological parameters and identify key active ingredients and targets. Additionally, the effects of Guilu Erxian gum on osteogenic differentiation of bone marrow mesenchymal stem cells were evaluated through alkaline phosphatase assay and alizarin blue calcium nodule staining. The expression of osteogenesis-related genes (Runx2, Osterix, OPN) was assessed using RT-PCR and Western blot. Results: The analysis revealed that Guilu Erxian gum contained 41 active ingredients and 236 targets, while there were 5,262 known therapeutic targets for osteoporosis. A protein-protein interaction network diagram was constructed using the String database. Enrichment analysis suggested that Guilu Erxian gum may regulate osteoporosis through various signaling pathways and cellular metabolism. The experiments demonstrated that Guilu Erxian gum could promote osteogenic differentiation of bone marrow mesenchymal stem cells, potentially contributing to the treatment of osteoporosis. Moreover, the results showed that Guilu Erxian gum could increase the expression of osteogenesis-related genes (Runx2, Osterix, OPN). Conclusion: Guilu Erxian gum may have anti-osteoporosis effects by stimulating the osteogenic differentiation of stem cells. This study provides a theoretical basis for further understanding the pharmacological mechanism of Guilu Erxian gum in treating osteoporosis.

Keywords: Chinese medicine; network pharmacology; Guilu Erxian gum; osteoporosis; mechanism of action
In this study, we used network pharmacology to study the mechanism of Guiliu Erxian gum in the treatment of osteoporosis, and at the same time, for the experimental verification of the results of the study, the results showed that Guiliu Erxian gum has a certain preventive and therapeutic effect on osteoporosis.

Medical history of objective
Guiliu Erxian gum is a classic prescription for the treatment of osteoporosis. Guiliu Erxian gum is contained in the “Yibian” (1587 C.E.) written by Wang Sancai in the Ming Dynasty. In ancient times, Guiliu Erxian gum was commonly used in the treatment of osteoporosis. Modern pharmacological studies have shown that Guiliu Erxian gum has the effects of protecting osteoblasts, promoting osteogenic differentiation of stem cells, inhibiting inflammatory factors, and anti-osteoarthritis.

Background
Osteoporosis (OP) is a condition that affects the bones throughout the body, resulting in a decrease in bone mass and damage to the structure of the bone tissue. This causes the bones to become more fragile and increases the risk of fractures [1, 2]. Globally, more than 200 million women aged 60 and above suffer from OP, and over 9 million people experience OP-related fractures annually [3]. Current research on OP pathogenesis has centered on cell differentiation and apoptosis, osteogenesis/osteolysis-related signaling pathways, and gene polymorphisms [4]. Western medical treatments mainly employ bone resorption inhibitors, bone formation stimulants, and bone mineralization drugs. Nevertheless, many of these drugs have significant side effects and potential drug interactions. For instance, long-term use of hormonal drugs can increase the risk of breast cancer and cardiovascular events [5]. Consequently, further optimization of clinical OP treatment and management is imperative.

The complexity of Chinese medicines and their compounds, characterized by multifactorial, multi-pathway, and multitarget synergistic effects, poses challenges in conducting comprehensive and systematic studies spanning macroscopic to microscopic levels. These challenges stem from the unclear material basis of the efficacy of Chinese medicines, obscure mechanisms of action, difficulty in controlling the quality of Chinese medicines and herbal materials, and the lack of a scientific, rational, and effective evaluation system for efficacy and safety [6]. Guiliu Erxian gum, a traditional Chinese medicine formulation prescribed by Ming Dynasty physicians, represents a typical product with a variety of ingredients, mainly derived from processed tortoiseshells and antlers. For centuries, Guiliu Erxian gum has been widely used in China for treating and preventing osteoporosis with minimal side effects. Modern pharmacological studies have demonstrated that the extract of Guiliu Erxian gum enhances the production of BMP-2 in osteoblasts, contributing to its preventive and therapeutic effects on osteoporosis. In recent years, network pharmacology has gained global recognition as a field that combines techniques and concepts from systems biology, multidirectional pharmacology, computational biology, and network analysis. It allows for the creation of complex networks and enables the exploration of the connection between drugs and diseases from a comprehensive viewpoint [7, 8]. Network pharmacology offers a new approach to investigating the active ingredients and targets of traditional Chinese medicine (TCM) compounds at a systemic level, which is particularly useful in understanding the multicomponent and multi-target relationship of TCM. This methodology aligns with the belief that Chinese medicine can regulate the body as a whole and exert therapeutic effects holistically. Thus, this study employs a network pharmacology approach to analyze the underlying materials that contribute to the efficacy of Guiliu Erxian gum in treating osteoporosis and to explore its potential molecular mechanisms of action. The goal of the study is to establish the connection between “active inglueulent-target-pathway” and investigate the multiple components, targets, and pathways involved in the formula’s effectiveness for treating osteoporosis. The findings will serve as a foundation for further basic experimental research on osteoporosis and provide a theoretical basis for the rational clinical application of Guiliu Erxian gum.

Materials
Herbal compounds and targets in Guiliu Erxian gum
The compounds present in Ginseng Radix, Lycii Fructus, tortoise plate, and antler gum in Guiliu Erxian gum were identified using traditional Chinese medicine system pharmacology technology platforms, including TCMP (http://tcmspw.com/tcmsp.php), the ETCPM database (http://www.tcmip.cn/), the chemistry database (http://www.organchem.csdb.cn/), and the DrugBank database (https://www.drugbank.ca/). The compounds found in Ginseng Radix and wolfberry were retrieved from the TCMP database, while those in tortoise plate and antler gum were not available in TCMP. Therefore, they were searched in the BATMAN-TCM database and the chemical professional database. For screening the compounds based on drug ADME (absorption, distribution, metabolism, excretion) characteristics, oral bioavailability (OB), and drug-like properties (DL) were used as the criteria, with a screening threshold of OB ≥ 30% and DL ≥ 0.18. However, after consulting the literature, it was discovered that tortoiseshell contains compounds that can promote the differentiation of mesenchymal stem cells (MSCs) into osteoblasts, while antler gum contains various amino acids and compounds that can enhance calcium absorption and bone mineralization. As a result, these compounds were considered as the active components of tortoiseshell gum and antler gum for further analysis.

Potential targets of Guiliu Erxian gum
The TCMP database was utilized to match the active components present in tortoiseshell gum, antler gum, Ginseng Radix, and Lycii Fructus with their corresponding potential targets. Each protein’s name was inputted using the UniprotKB search function (http://www.Uniprot.org/) in the Uniprot database, with the species specified as “human”. The searched proteins were then cross-referenced with their official names, ensuring that they represented the predicted targets of the active components.

Screening of disease targets
The OMIM database (https://omim.org/), Disgenet database (https://www.disgenet.org/), GeneCards database (https://www.genecards.org/), and CTD database (http://ctdbase.org/) were queried using “osteoporosis” as the keyword to retrieve the disease target information.

Interaction network between active component targets of Guiliu Erxian gum and OP-related therapeutic targets
The outcomes of the active component genes and OP disease genes from Guiliu Erxian gum were entered into the online Venn diagram platform, Wayne Venn map (http://bioinfogp.cnb.cscic.es/tools/venny/index.html), to identify the intersecting genes and create the Wayne map. Subsequently, the intersecting genes were analyzed using the STRING platform (https://string-db.org/) to map the known therapeutic targets of OP to the targets of the active components found in Guiliu Erxian gum from tortoise and deer. The analysis was performed on Homo sapiens as the chosen species, and the unlinked proteins were concealed to construct the protein-protein interaction network (PPI). The PPI protein interaction map was downloaded, and the top 30 core genes from the PPI network were selected and utilized to generate the bar chart.

“Drug-ingredient-target” network construction
The intersection target genes obtained previously were imported into
Cytoscape 3.8.0 software, and network topology analysis was conducted using the tools correlation analysis plug-in to construct a display map of the network structure involving the direct or indirect target gene proteins influenced by Guilu Erxian gum in osteoporosis treatment. Based on the network topology analysis plug-in, nodes with node connectivity (degree) more than twice the median of all nodes were selected. Furthermore, nodes with other indices larger than the median of all nodes were screened to identify key target genes (hubs) that play a significant role in the direct or indirect regulation of osteoporosis treatment.

Gene ontology (GO) function and KEGG pathway enrichment analysis

The bioconductor biology data package of R4.0.2 was employed to perform GO function and KEGG pathway enrichment analyses. Statistical significance for both GO biology enrichment analysis and KEGG pathway enrichment analysis was determined using a p-value threshold of 0.05 and a q-value of 0.05. Subsequently, the study elucidated the therapeutic mechanism for osteoporosis.

Molecular docking

Using the Cytoscape software plug-in, a “drug-component-target-disease” network graph was computed. The top three active components based on the degree ranking were subjected to docking with the top three proteins in the PPI network graph. The structural formulae of the active components were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov), and their corresponding 3D structures were generated using Chem3D software. These structures were then exported to the mol2 format. Additionally, the core proteins were retrieved from the PDB database (http://www.rcsb.org/) in PDB format. The PDB format of the structural domains was also acquired from the PDB database. Subsequently, the protein structures underwent de-watering and de-phosphorylation processes using PyMOL software. The PDB format of the active ingredient and core protein gene files was converted to pdbqt format using AutoDockTools 1.5.6 application software. Finally, the Vina script was executed to carry out molecular docking energy calculation and molecular docking. The resulting output of molecular docking was imported into PyMOL software for visualization and analysis.

Experimental verification

Experimental animals. This study utilized 50 female Sprague-Dawley (SD) rats who were healthy and one month old as the experimental animal model. The rats were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, and were kept in a controlled environment with specific pathogen-free (SPF) conditions. This study followed the National Institutes of Health Laboratory Animal Care and Guidelines, and was approved by the Experimental Animal Ethics Committee of Hubei Provincial Hospital of Traditional Chinese Medicine (ethics approval number: No. 20220149).

Pharmacological substances. Guilu Erxian gum, a therapeutic formulation, comprised 9 g of antler gum, 6 g of tortoise plate gum, 6 g of Ginseng Radix, and 6 g of Lycii Fructus. A concentrated granule form of Guilu Erxian gum (11.55 g) was obtained from Hubei Traditional Chinese Medicine Pharmacy and transferred into a calibrated reagent bottle with a blue lid. Distilled water was added to attain a volume of 30 mL in accordance with the specified scale. This preparation yielded a solution of Guilu Erxian gum, with a concentration of 1 g/mL of crude drug. To facilitate the complete and uniform dissolution of particles, the Guilu Erxian gum solution underwent continuous stirring at a constant temperature of 36 °C. Precautions were taken to ensure the sterilization of the reagent bottles, intragastric needles, and other utensils through the application of high-pressure sterilization techniques.

Preparation of drug-containing serum derived from Guilu Erxian gum. A total of 40 healthy female SD rats, aged three months and adhering to the SPF grade, were randomly allocated to four groups: high dose, middle dose, low dose of Guilu Erxian gum, and a blank control group, each consisting of 10 rats. The daily dosage for each rat was determined based on the dose conversion coefficient table for humans and rats, using the formula (d) = 30 g/60 kg × 6.3 × rat-specific weight (kg). Therefore, the equivalent doses were as follows: 1.575 g/kg/d was determined for the low dose, 3.15 g/kg/d for the medium dose and 6.3 g/kg/d for the high dose. The low, middle, and high doses of Guilu Erxian gum decoction were administered through gastric perfusion, while the blank control group received an equal volume of normal saline. Additionally, a positive control group underwent daily gastric perfusion for seven consecutive days. Within a two-hour timeframe following the final administration, blood samples were collected through the abdominal aorta under sterile conditions. The collected samples were left at room temperature for three hours, followed by overnight refrigeration at 4 °C. Subsequently, the samples were centrifuged at a rate of 2,500 r/min for 20 minutes to collect the supernatant. All serum samples were activated at 56 °C for 30 minutes, aseptically filtered, and then stored at −20 °C. The serum derived from each group was utilized within one month after preparation.

Extraction and culture of bone marrow mesenchymal stem cells (BMSGs).

The rats were euthanized through cervical dislocation and immersed in 75% ethanol for five minutes. Under sterile conditions, the bilateral femurs were separated on a sterile worktable, ensuring the removal of any attached muscle tissue. The femurs were thoroughly washed with Phosphate Buffered Saline(PBS) containing 1% dual antibodies. The epiphyses were severed, exposing the bone marrow cavity, and an aseptic syringe (5 mL) was used to aspirate the Dulbecco’s modified eagle medium (DMEM) culture medium. The marrow cavity was repeatedly rinsed to obtain a single-cell suspension. The mixed cell suspension that was obtained underwent centrifugation at 1,000 rpm for 5 minutes, and the liquid part above the solid was discarded. The cells that settled at the bottom were then mixed again in a DMEM culture medium with 10% fetal bovine serum. After passing through a filter that had a mesh size of 200 and had been sterilized, the cells were placed in a culture bottle that had a size of 25 cm² and was incubated in an incubator that had a temperature of 37 °C and a CO₂ concentration of 5%. After 24 hours, half of the culture medium was replaced once the cells had been added, and any further changes to the medium were made every two days. The cells’ growth status was monitored using an inverted phase contrast microscope. Upon reaching approximately 80% confluence (8–12 days), trypsin solution (0.25%) was used for cell detachment and passage. For the first passage, the cells were transferred to new 25 cm² flasks at a 1:2 ratio, and daily observations were made using an inverted phase contrast microscope. The process of cell passage was repeated whenever cell confluence was reached, ensuring that the cells were subcultured into new 25 cm² culture bottles at a 1:2 ratio. Experimental grouping interventions were carried out following these passages.

Observation indicators and methods

To accustom the rats to the laboratory setting, a total of 40 healthy male SD rats with SPF status were housed in the laboratory for one week. Following this period, they were randomly divided into four groups: the control group, low-dose Guilu Erxian gum group, medium-dose Guilu Erxian gum group, and high-dose Guilu Erxian gum group. The control group rats were given a dosage of 10 g/kg/d of ultra-pure water for seven weeks. After the final administration, the rats fasted for 12 hours with access to water. They were then anesthetized using intraperitoneal injection of pentobarbital sodium solution, and blood was collected from the abdominal aorta to obtain serum. The drug-containing serum, as well as BMSCs, were culpable for further experiments. The positive control group was treated with a conventional osteogenic inducer consisting of DMEM culture medium, 10% fetal bovine serum, 50 g/mL vit. C, 10 mmol/L glycerol phosphate, and 10 mmol/L dexamethasone.

In order to assess the therapeutic effects of Guilu Erxian gum on osteoporosis, we randomly divided BMSCs from the P3 generation, submitted a manuscript: https://www.tmrmagazines.com/tmr
which showed optimal activity, into five groups: a control serum group, two groups treated with classical osteogenic inducers, a group with low-dose drug-containing serum, and a group with medium-dose drug-containing serum. The cells were placed in a 6-well plate at a density of $3.5 \times 10^5$ cells/mL and cultured accordingly. After seven days of induction, the culture medium was removed, and the cells were fixed with anhydrous ethanol. Subsequently, the cells were washed with PBS for 5 minutes per wash. A staining solution was then applied, and the cells were stained at room temperature, protected from light, for at least 30 minutes until color development occurred blue. After staining, the dye solution was removed, and the cells were washed twice with double-distilled water to stop further color development. A nuclear solid blue dye solution was added for subsequent staining. The cells were dehydrated with anhydrous ethanol, made transparent with xylene, and allowed to dry in a ventilated cabinet. Finally, the slides were sealed with neutral gum, and the optical density was measured using IPP software for semi-quantitative analysis.

The grouping procedure was maintained in line with the previous section. BMSCs from each group were plated onto a 6-well plate at a concentration of $3 \times 10^5$ cells/mL. Once the cells reached 70% to 80% confluence, osteogenesis induction was started in each respective culture medium. The culture medium was regularly renewed, and after 21 days, staining for osteogenesis was carried out. To prepare for staining, the cells were removed from the incubator, and the original culture medium was carefully aspirated without disturbing the cell colony at the bottom. Next, 1 PBS was added slowly to each well, followed by three washes. The cells were then fixed with 4% paraformaldehyde, with 1 mL of fixed solution added to each well. The plate was gently agitated to ensure uniform coverage of the fixative solution and left to fix for 15 minutes at room temperature. The fixative solution was then removed while taking care to prevent cell detachment, and the wells were rinsed with double-distilled water. The timing for staining and rinsing was consistent among all groups. Finally, the plate was air-dried, and the calcium nodules were observed and photographed using an inverted microscope. The density of calcium nodules was then analyzed semi-quantitatively using ImageJ software.

The high-quality P3 generation BMSCs were cultured on a 6-well plate. Once the cell confluence reached 80%, the cells were divided into five groups: a control group without serum, a group subjected to classical osteogenic induction, and groups treated with low, medium, and high-dose drug-containing serum. The culture medium was changed every 48 to 72 hours. After 21 days of continuous culture, RT-PCR detection was performed. Using actin as the internal reference, Ct values were noted, and the expression levels of osteogenesis-related genes (Runx2, Osterix, OPN) were calculated using the $2^{\Delta\Delta Ct}$ method.

The experimental groupings were divided into 6 groups as before. To gain deeper insights into the mechanism of Guiliu Erxian gum on the osteogenic differentiation of BMSCs, we examined the expression of osteogenesis-related proteins (Runx2, Osterix, OPN) after 21 days of culture using Western blotting. Subsequently, we compared the differences between the experimental groups.

**Results**

**Compounds and targets of Guiliu Erxian gum**

Utilizing the TC MSP database, a total of 22 active constituents were identified from *Ginseng Radix* and 43 active constituents from *Lycium barbarum*, employing the criteria of OB $\geq 30\%$ and DL $\geq 0.18$. Subsequently, these active constituents were systematically linked to their respective potential targets using the TC MSP database. As a result, non-target active constituents were excluded, leading to the identification of 181 targets for *Ginseng Radix*, 109 targets for *Lycium Practus*, 265 targets for tortoise shell, and 28 targets for antler. The entire formulation encompassed a collective total of 236 targets. To ensure accuracy and eliminate redundancies, the Uniprot database was employed to rectify the target names to their official nomenclature and remove any duplicates. This meticulous process resulted in the determination of 41 active constituents and 236 targets for Guiliu Erxian gum, as detailed in [Supplementary Table 1–4](https://doi.org/10.53388/TMR20230704002).

**Screening and collection of disease target information**

We collected a total of 5,861 therapeutic targets associated with OP from the OMIM database, Dinagenet database, Genecards database, and CTD database. After eliminating duplicates, we identified 5,262 targets in total (refer to [Supplementary Table 5](https://doi.org/10.53388/TMR20230704002) for further information). The active ingredient targets of Guiliu Erxian gum and the target genes of OP were inputted into an online Venn diagram platform to generate Venn diagrams. The Venn diagrams depicted the common targets corresponding to the key compounds of osteoporosis, while irrelevant compounds were excluded. Through this process, a final set of 160 common targets associated with Guiliu Erxian gum and

### Table 1 Primer sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>PCR products</th>
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<tr>
<td></td>
<td>Reverse</td>
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<td></td>
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<tr>
<td>Rat RUNX2</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
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</tr>
<tr>
<td>Rat Bmp2</td>
<td>Forward</td>
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<td>139bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCGCTGTGTGTTGTTTG</td>
<td></td>
</tr>
<tr>
<td>Rat Osterix</td>
<td>Forward</td>
<td>GAAAAGGAGCCACAAAGAG</td>
<td>174bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGGAAAATGAGTGGGGAGAAG</td>
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<tr>
<td>Rat β-catenin</td>
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<td></td>
<td>Reverse</td>
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<tr>
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<td></td>
<td>Reverse</td>
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osteoporosis were obtained. The results are visually presented in Figure 1. Further details on the intersecting targets can be found in Supplementary Table 6.

**PPI network construction**

The common target information of drugs and diseases was queried in the STRING database, and the results were exported upon completion of the setup. Subsequently, the obtained results were imported into the Cytoscape software system to generate a network diagram illustrating protein interactions (Figure 2). In this diagram, the nodes represent individual protein-coding genes, and the connections between nodes indicate protein network interactions between two genes. The top 10 genes based on degree were JUN, RELA, AKT1, HSP90AA1, MAPK1, TNF, FOS, ESR1, IL6, and MAPK14. These findings suggest that these ten target proteins are central nodes in the PPI network and are potential key targets for intervention in osteoporosis using Guilu Erxian gum. The results are depicted in Figure 3.

"Drug-component-target-disease" network diagram construction

The identified overlapping genes were utilized in the construction of the visualization network for Guilu Erxian gum-Osteoporosis using Cytoscape 3.8.0 software (Figure 4). The network consists of 123 nodes, encompassing 71 genes, 50 key compounds, one disease name, and one drug name. Within the network, the red rectangles represent the 50 crucial active ingredients, including steroids and flavonoids.
The blue diamond shape corresponds to the 71 overlapping genes. The chemical components ranked highest in terms of node degree are as follows: quercetin (86), beta-sitosterol (11), and glycine (11).

**GO and KEGG pathway enrichment**

In order to understand the underlying therapeutic effects of Tortoise and Guili Erxian gum on osteoporosis, a GO analysis was conducted on 160 common targets. This analysis investigated the biological processes (BP), cellular components (CC), and molecular functions (MF) associated with these targets. The most significant BP, CC, and MF terms, identified through P values, are presented in Figure 5. Additionally, a detailed GO enrichment analysis for BP, CC, and MF can be found in Supplementary Table 7–9. Furthermore, a KEGG pathway enrichment analysis was performed to further explore the potential mechanism of Guili Erxian gum in treating osteoporosis. The results, provided in Supplementary Table 10, revealed that 160
therapeutic targets were associated with 255 KEGG pathways, with a significance level of $P < 0.05$. Figure 5 illustrates the top 20 matches among these pathways. Notably, these 106 KEGG pathways are involved in various human diseases, signaling pathways, and pathophysiological mechanisms. The most significant signaling pathways, based on their $P$-values, include the AGE-RAGE signaling pathway in diabetic complications, lipid and atherosclerosis, fluid shear stress and atherosclerosis, chemical carcinogenesis-receptor activation, prostate cancer, hepatitis B, IL-17 signaling pathway, TNF signaling pathway, pancreatic cancer, and small cell lung cancer. These findings suggest that Guilu Erxian gum may have therapeutic effects on osteoporosis by influencing key targets within these signaling pathways and that the majority of therapeutic targets are involved in multiple signaling pathways.

Molecular docking
The docking process of the primary active ingredient (quercetin) and the target proteins (JUN, RELA, AKT1) was conducted using Autodock Tool 1.5.6 to elucidate the binding efficacy. According to Table 2, the binding energy between quercetin and the target proteins range from $-5.01$ to $-5.96$ kcal/mol$^{-1}$. Molecular analysis further confirmed that the binding energy of quercetin to the proteins was below $-5$ kcal/mol$^{-1}$ [9], as presented in Table 2. These results indicate the stability of the molecular binding between the active ingredient and the target proteins. The docking conformations of the top three positions were analyzed using PyMOL and Photoshop, illustrating the stable binding of quercetin to JUN, RELA, and AKT1 (Figure 7).

Experimental verification
Alkaline phosphatase detection. After a week of introducing the culture medium, we observed blue-purple stained areas of varying depths under the microscope in all groups of petri dishes. The lowest optical density was found in the group treated with blank serum, while the groups treated with drug-containing serum and osteogenic induction displayed significantly higher optical densities comparable to the blank serum group ($P < 0.01$). Additionally, the optical density of the drug-containing serum groups increased as the dosage increased, leading to a larger stained area. Although the optical density in the osteogenic induction group was higher than that in the high-dose drug-containing serum group, no statistically significant difference was observed ($P > 0.05$). Therefore, the results indicate that Guilu Erxian gum enhances the osteogenic differentiation capacity of BMSCs, particularly in the high-dose group. For a visual representation, please refer to Figure 8.

Staining and quantification of alizarin red mineralized nodules.
On the 21st day after BMSC induction in each culture medium group, the staining results of alizarin blue calcium nodules revealed that a significant amount of orange-stained calcified nodules were present in all groups, excluding the blank serum group. In contrast, only a few scattergun calcified nodules were quantity of calcified observed in the blank serum group, with minimal blue staining. The number of calcified nodules in the drug-containing serum groups and the osteogenic induction group exhibited a significant increase comparable to the blank serum group ($P < 0.01$). Additionally, the modules was directly proportional to the dose of Guilu Erxian gum. Among the drug-containing serum groups, the high-dose group displayed the highest number of calcified nodules, while the osteogenic induction group demonstrated a higher count than the high-dose group, although not statistically significant ($P > 0.05$).
Table 2 Binding energy of key active ingredients to core proteins

<table>
<thead>
<tr>
<th>Compound</th>
<th>JUN</th>
<th>RELA</th>
<th>AKT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>-5.01</td>
<td>-5.96</td>
<td>-5.61</td>
</tr>
</tbody>
</table>

Figure 6 A histogram barplot illustrates the enrichment of key target signaling pathway in the treatment of osteoporosis by Guliu Erxian gum. The name of the pathway is represented on the vertical axis, while the enrichment factor is shown on the horizontal axis. A greater enrichment factor suggests a higher level of significance for the target in the pathway. The color of the bubble becomes darker blue as the $P$ value of the enrichment result decreases, indicating greater reliability. Furthermore, the size of the bubble corresponds to the number of genes that are enriched in the pathway, with larger bubbles indicating a higher number of enriched genes, and vice versa.

Figure 7 Molecular docking result diagram
Figure 8 Displays the alkaline phosphatase staining and semi-quantitative analysis of BMSCs (crystal violet staining × 100). (A) Blank group. (B) Positive control group. (C) Low dose group. (D) Middle dose group. (E) High dose group. (F) Integrated optical density (× 10²). According to the findings, the blank group had significantly lower optical density comparable to the other groups. Furthermore, although the difference was not statistically significant (\( P > 0.05 \)), the high dose group had lower optical density comparable to the inducer group. Additionally, the results indicated that the optical density in the drug-containing serum group increased as the dose increased. BMSCs, bone marrow mesenchymal stem cells.

Please refer to Figure 9 for a visual representation.

**Determination of expression of osteogenesis-related genes by RT-PCR.** After 21 days of osteogenic induction intervention, the expression of osteogenesis-related genes (Runx2, Osterix, OPN) in the BMSCs of each group was determined. The relative expression levels of Runx2, Osterix, and OPN in BMSCs of the osteogenic induction group and each dose of Guilu Erxian gum-containing serum group were significantly higher comparable to the blank serum group (\( P < 0.01 \)). As the dose of the drug-containing serum increased, the relative expression levels of Runx2, Osterix, and OPN mRNA in the low, middle, and high-dose groups showed a progressive increase. The high-dose drug-containing serum group exhibited the most significant increase in the relative expression of Runx2, Osterix, and OPN mRNA. However, when comparable to the osteogenic induction group, the relative expression of Runx2 and Osterix mRNA in the high-dose drug-containing serum group was slightly lower, although the difference was not statistically significant (Figure 10).

**Determination of the expression of osteogenesis-associated proteins by Western Blot.** Following osteogenic induction of BMSCs in each culture medium for 21 days, the expression of osteogenesis-related proteins in BMSCs was ascertained. Comparable to the control serum group, the osteogenic induction group and each dose of the drug-containing serum group exhibited a significant increase in the expression of Runx2, Osterix, and OPN proteins (\( P < 0.01 \)). Furthermore, when compared to BMSCs treated with a high dose of Guilu Erxian gum drug-containing serum, the protein expressions of Runx2, Osterix, and OPN in BMSCs of the middle and low-dose Guilu Erxian gum drug-containing serum groups were found to be lower (\( P < 0.05 \)). The findings from ALP staining, ALP activity assay, mineralized nodule staining, and osteogenesis-related gene detection collectively indicate that there exists a dose-effect relationship between the osteogenic differentiation activity of BMSCs and the effect of Guilu Erxian gum. Specifically, the increase in osteogenic differentiation activity is positively associated with the dose of Guilu Erxian gum with the high-dose serum containing Guilu Erxian gum exhibiting the most optimal effect. Refer to Figure 11, 12 for further details.

**Discussion**

With the aging of the global population, osteoporosis prevalence is increasing. Modern medicine attributes the incidence of osteoporosis to disturbances in bone metabolism, where bone resorption surpasses bone formation, resulting in decreased bone mineral density per unit volume and eventually leading to osteoporosis. Current clinical drug treatments targeting osteoporosis have limited effectiveness due to their multifactorial etiology. Traditional Chinese medicine, on the other hand, views the pathogenesis of osteoporosis as related to kidney essence deficiency and offers advantages in treating diseases through multifaceted and multitarget approaches, thus holding clinical value in osteoporosis treatment [10]. Previous studies have demonstrated that Guilu Erxian gum improves clinical symptoms and offers a protective effect on bone mineral density in osteoporosis patients [11]. However, the specific mechanism of its action remains unclear. This study aims to elucidate the molecular mechanism of Guilu Erxian gum in osteoporosis treatment using network pharmacology and molecular docking technology, providing a theoretical foundation for its application in traditional Chinese medicine-based osteoporosis treatment.

The key targets of Guilu Erxian gum in osteoporosis treatment include JUN, AKT1, MAPK1, TNF, FOS, ESR1, IL6, MAPK14, and others. JUN, a member of the AP-1 transcription factor family, plays a crucial role in regulating gene expression related to bone marrow mesenchymal cells and osteoblasts. It promotes osteoblast proliferation, differentiation, and bone tissue repair, thus repairing bone defects [12, 13]. AKT1, a threonine protein kinase, is an intermediary in osteoblast and osteoclast differentiation signal transduction, facilitating the differentiation of bone marrow mesenchymal cells into osteoblasts [14]. MAPK1 is involved in the PI3K-AKT signaling pathway in osteoporosis treatment, and PI3K-AKT is an apoptosis signal pathway regulating the expression of apoptosis-related protein DE [15, 16]. IL-6 is closely associated with osteoporosis pathogenesis, and its increased serum levels are a significant risk factor for osteoporosis development. IL-6 promotes osteoclast formation and bone resorption by stimulating osteoblasts to release IL-1 and prostate E2, reducing OPG expression and increasing RANKL.
Figure 9 illustrates the formation and semi-quantitative analysis of calcified nodules derived from BMSCs in each respective group (alizarin red staining, magnification × 100). (A) Blank group. (B) Positive control group. (C) Low dose group. (D) Middle dose group. (E) High dose group. (F) Alizarin red area ratio(%). The findings revealed that the blank group had a noticeably lower amount of calcified nodules compared to the other groups (* P < 0.01). Furthermore, while the high dose group had a lower number of calcified nodules than the inducer group, this difference was not statistically significant (P > 0.05). Moreover, the number of calcified nodules in the drug-containing serum group increased as the dose increased. BMSCs, bone marrow mesenchymal stem cells.

Figure 10 mRNA expression of osteogenic proteins. (A) Relative expression of Runx2 mRNA. (B) Relative expression of Osterix mRNA. (C) Relative expression of OPN mRNA. Compared with the blank group, ** P < 0.01; compared with the high dose group, * P < 0.05, † P < 0.01, ‡ P < 0.01; compared with the positive control group, * P < 0.01.

Figure 11 Western blot results of osteogenic associated proteins. (A) Relative expression of Runx2 protein. (B) Relative expression of Osterix protein. (C) Relative expression of OPN protein. Compared with the blank group, ** P < 0.01; compared with the high-dose group, ** P < 0.01, † P < 0.01, ‡ P < 0.01; compared with the positive control group, * P < 0.01.
expression [17–19].

Based on the "drug-disease-component-target" network analysis, querectin, kaempferol, and naringen emerge as the most active chemical components related to osteoporosis in Guili Erxian gum. Querectin, for instance, can promote bone marrow mesenchymal stem cell proliferation and osteogenic differentiation via the Wnt/β-catenin signal pathway while inhibiting osteoclast-mediated bone resorption [20]. β-sitosterol, the main phytosterol in plants, is also believed to have an anti-osteoporotic effect. Animal experiments suggest that it reduces bone resorption markers and increases osteogenic markers by protecting osteoblasts and inhibiting osteoclast formation, partly through the regulation of RANKL/OPG and RunX2 pathways [21]. Additionally, glycine and other amino acids in deer bone powder have been shown to increase bone mineral density and regulate bone mass and microstructure [22, 23].

The GO enrichment analysis indicates that the target genes are mainly involved in processes such as antioxidation, stress, active oxygen metabolism, and regulation of inflammatory response. The KEGG pathway analysis highlights the importance of the AGE-RAGE signaling pathway in diabetic complications, IL-17 signaling pathway, and TNF signaling pathway, among others, in Guili Erxian gum's treatment of osteoporosis. The AGE-RAGE signaling pathway creates an inflammatory environment, activating the NF-κB signal pathway and inducing oxidative stress response, contributing to tissue degeneration [24]. Similarly, the IL-17 signaling pathway plays a role in human immunity and chronic inflammation, promoting inflammatory effects through the activation of transcription factors and the release of various inflammatory factors [25, 26]. Guili Erxian gum is believed to control inflammatory responses and promote osteogenic differentiation of stem cells, potentially slowing down bone degeneration and loss [27, 28].

In conclusion, this study employed network pharmacology to integrate molecular computing, network analysis, and text-mining techniques. The investigation focused on the chemical composition, action targets, and related disease signal pathways of Guili Erxian gum using a multitarget, multidirectional, and multi-level approach. The results provided preliminary verification of the material basis and mechanism underlying the preventive and therapeutic effects of Guili Erxian gum on osteoporosis. The study not only highlighted the characteristics of multi-components, multi-targets, and synergism but also established a solid foundation for further mechanistic research. However, network pharmacology as a novel drug research method has its limitations. Two main shortcomings were identified in this study. Firstly, the metabolic absorption process of Guili Erxian gum in the human body remains undetermined, and relevant toxicological detection for potential side effects is lacking. Hence, further experiments will be conducted to ascertain possible side effects. Secondly, Guili Erxian gum has not been compared with current clinical first-line anti-osteoporosis drugs, resulting in a lack of assessment regarding its related clinical effects. Consequently, future clinical studies will be conducted to compare Guili Erxian gum with these drugs. At present, the osteogenic effect of Guili Erxian gum is primarily established through experimental evidence, suggesting its potential anti-osteoporosis efficacy. However, there is a need for further research on its effective active components and related mechanisms, which will be pursued in future investigations.

References


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