Spectrum-effect relationship between HPLC fingerprints and bioactive components of *Radix Hedysari* on increasing the peak bone mass of rat

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**Abstract**

The traditional Chinese medicine of *Radix Hedysari* plays an important role in invigorating gas for ascending, benefiting blood for promoting production of fluid, and promoting circulation for removing obstruction in collaterals, which is consistent with the principle of treatment for osteoporosis. This study is designed to investigate the bioactive components on increasing peak bone mass (PBM) by exploring the spectrum-effect relationship between chromatography fingerprints and effect. Multiple indicators are selected to evaluate the pharmacological activity. In fingerprints, 21 common peaks are obtained, five of which are identified. Furthermore, gray relational analysis (GRA) is a quantitative method of gray system theory and is used to describe the correlation degree of common peaks and pharmacological activities with relational value. 21 components are then divided into three different regions, of which ononin and calycosin play an extremely significant role in increasing PBM. In addition, factor analysis and hierarchical cluster analysis (HCA) are used to screen the optimal producing area for *Radix Hedysari*. This provides a comprehensive and efficient method to improve the quality evaluation of *Radix Hedysari*, confirming the bioactive components for PBM-enhancement and further develop its medicinal value.

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**Keywords:**
- Factor analysis
- Gray relational analysis
- Hierarchical cluster analysis
- Peak bone mass
- *Radix Hedysari*
- Spectrum-effect relationship

**1. Introduction**

Traditional Chinese medicine (TCM) has attracted international attention in recent years due to its complementary curative effects to western medicines [1]. Considering the complexity and diversity of TCM components that induce multiple targets and relate to kinds of effect, a change from traditional evaluation methods emphasizing chemical components to a method focusing on biological effects is necessarily required [2]. The spectrum-effect relationship is used to explore the bioactive components of TCM combined with a variety of statistical methods, such as regression analysis, cluster analysis, principal component analysis, gray relational analysis, discriminant analysis, and neural network analysis.

Chromatographic fingerprint is an efficient method for the identification and quality control of TCM [3]. The high performance liquid chromatography method coupled with diode array detection (HPLC-DAD) is developed to characterize the components of TCM. Considering the complexity of TCM, the different polar extracts are obtained using solvents with various polarities, which play different roles on the human body. The ingredients are accurately identified by comparing the retention time and UV absorption between reference substances and samples. Furthermore, the bioactive components are screened out by exploring the relationship of fingerprints and the effect.

Osteoporosis is a chronic, progressive disease of skeleton characterized by loss of bone tissue and deterioration of osseous microarchitecture, leading to increased risk of fragility fracture [4,5]. Peak bone mass (PBM) is the peak amount of bone acquired by one when he is mature, which degreases gradually after maturating stage. Bone amount can be reckoned as the difference between PBM and bone loss [6]. PBM is the basic standard for the diagnosis of osteoporosis. The basic theories of TCM demonstrate that clinical manifestations of osteoporosis should belong to the category of "paralysis of bone", "bone atrophy", "lumbago", and "fracture" [7]. It is believed that kidney essence deficiency, hepatic depression syndrome and spleen-stomach deficiency are three crucial factors causing osteoporosis [8].

*Radix Hedysari*, known as "HongQi" (HQ) in Chinese, is the main roots of *Hedysarum polybotrys* Hand.-Mazz and a species belonging to the fabaceae family [9]. In China, the producing areas for *Radix Hedysari* are mainly in Gansu, Sichuan and Inner Mongolia. *Radix Hedysari* is a well-known Chinese herbal medicine used for the treatment of diarrhea, diabetes, chronic nephritic proteinuria, inflammation and the immune-enhancement [10] due to the various effective ingredients, such as polysaccharide, flavonoids, ginsenoside, trace elements and amino acids [11]. Many modern
researches have focused on the pharmacodynamics of *Radix Hedysari* in recent years. Sun et al. [12] explored its effect on suppressing lipid metabolism dysfunction. Wei et al. [9] found its antioxidant activity and neuroprotective effects. Yu et al. [13] discussed its effect on inhibiting endotoxin-induced uveitis. Wei et al. [14] found its antitumor activities and antioxidant activity in vitro. However, a few literatures focused on sieving active ingredients of *Radix Hedysari* through spectrum relationship and there was no definite discipline to classify *Radix Hedysari*, which hinders the development of its medical value. In this study, we explored the effect of active components of *Radix Hedysari* in increasing the PBM and classified *Radix Hedysari* from different areas using statistical methods. According to the Chinese Pharmacopoeia (CHP 2010), *Radix Hedysari* plays an important role in invigorating gas for ascending, benefiting blood for promoting production of fluid, and promoting circulation for removing obstruction in collaterals [15], which is consistent with the principle of treatment for osteoporosis. It is crucial to explore bioactivity of *Radix Hedysari*, which is closely related to the quality assurance and security guarantees [16]. The study was thus designed to explore the relationship between fingerprints and the effect of *Radix Hedysari* on increasing PBM, in which the gray correlation degree method was used to explore the bioactive components, factor analysis and hierarchical clustering analysis were used to evaluate the similarity and variation of the samples. In conclusion, this study provides an efficient way in screening bioactive components of *Radix Hedysari* for improving osteoporosis and screening the optimal producing area for *Radix Hedysari*.

2. Experimental methods

2.1. Experimental

2.1.1. Animals

Female Sprague-Dawley (SD) rats (4–5 weeks old), weighing 100–120 g, were purchased from Animal Experimental Center of Lanzhou University (Qualification certificate number: SCXK (GAN) 2013-0002, Lanzhou, China). These animals were kept in a constantly controlled environment with rearing temperature at (24 ± 2) °C, humidity at (50%–55%) and a 12h dark-light illumination cycle for a week prior to the experiment. The diet and water were supplied ad libitum. All animal operations were carried out according to the rules of laboratory animal administration, and were approved by the Animal Ethics Committee of Lanzhou University, China.

2.1.2. Materials, reagents and instruments

The HPLC fingerprints of *Radix Hedysari* were performed using a Waters 2695 high performance liquid system, including 717 auto-sampler manager and 2996 UV detector, connected to Millennium 32 software (Waters, USA). Dual energy X-ray absorptionmetry (GE Prodigy, USA) was used to measure bone mineral density (BMD) rapidly and accurately. AG-X desktop electronic universal test machine (Shimadzu, Japan) was used to measure bone biomechanics as a convenient tool. Microplate reader (Bio-Rad 550, USA) was used to determine the spectral absorbance of samples.

Acetonitrile and pentyl barbital sodium were of analytical grade (Merck, Germany). Purified water was purchased from Wahaha Company (China). Reference standards of adenosine, calycosin–7-glucoside, ononin, calycosin, and formononetin were purchased from China drugs and biological products assay (Beijing, China, purity > 98%). Alkaline phosphatase (AKP), tartrate resistant acid phosphatase (TRACP), calcium (Ca), and phosphorus (P) were purchased from Jiancheng Biopharmaceutical Company (Nanjing, China).

2.1.3. Plant sources of Radix Hedysari

Ten batches of *Radix Hedysari* herbs from different origins were purchased from the markets of Chinese herb medicines or self-collected, and were authenticated as the dried roots of *Hedysarum Polybotrys Hand.-Mazz.* by Professor Zhigang Ma (School of Pharmacy, Lanzhou University, China). The sources of *Radix Hedysari* are shown in Table 1.

| No. | Sources                     |
|-----|----------------------------|
| 1   | Micang Mountain of WuDu     |
| 2   | Youzi Mountain of WuDu      |
| 3   | Shouyang Town of LongXi     |
| 4   | Caizi Town of LongXi        |
| 5   | Guanting Town of TanChang   |
| 6   | Nanyang Town of TanChang    |
| 7   | Chengguang Town of TanChang |
| 8   | Hadapu Town of TanChang     |
| 9   | Gangquan Town of WuDu       |
| 10  | Lichuan Town of TanChang    |

2.2. Experimental process

2.2.1. Preparation of different extracts from different polar parts of Radix Hedysari

The *Radix Hedysari* herb of No. 1 (Micang Mountain of WuDu) was weighed 500 g and extracted with 6-fold 95% ethanol for 3 times, 1 h each time. All of the extracted liquids were merged and vacuum recycled until there was no alcohol taste, 1/5 of which was concentrated until dryness to obtain the total extract of *Radix Hedysari* (TER). The residual solution was transferred to a separatory funnel, and extracted with an equal volume of petroleum ether and ethyl acetate for 5 times successively. As the methods described above, petroleum ether extract (PER) and ethyl acetate extract of *Radix Hedysari* (EAR) were obtained. The insoluble part was dissolved in ethanol and extract of *Radix Hedysari* (EER) was obtained after dryness. Besides, after ethanol extraction, the residue of *Radix Hedysari* was extracted by water decocting method with 10-fold hot water for 1.5 h once. The extracted fluid was vacuum concentrated and then centrifugated to get the supernatant, the precipitation of which was the crude polysaccharide of *Radix Hedysari* (HPS). According to the methods above, five extracts from different polar parts of *Radix Hedysari* were obtained [17].

2.2.2. Effects of different polar extracts on peak bone mass of rat

Rats were randomly divided into seven groups, 12 rats in each group, and administered by gavage once daily for consecutive three months with normal saline (NS) as sham group [18], XIAN-LINGUBAO (XLGB) tablet (0.6 mg/kg for one day) as positive group, and five extracts from different polar parts of *Radix Hedysari* (10 g crude drug/kg for one day) as drug groups. In the mid-stage of experiments, the bone mineral density of whole body (BMD-B) was detected by dual-energy X-ray absorptionmetry (GE Prodigy, USA) after 3% sodium pentobarbital was intraperitoneally injected into rats for anesthesia (0.1 mL/100 g). Three months later, the same testing indicators were detected. Furthermore, the orbital blood samples were obtained from rats for the detection of serum AKP, TRACP, Ca, and P using a microplate reader (Bio-Rad 550, USA). The bone mineral density of right femur (BMD-R) was also measured after the soft tissue was cleaned off [5,19]. The biomechanical parameters were detected by universal testing machine (DDL, German) using three-point bending method [20,21].
2.2.3. Effect of 10 TER samples from different origins in increasing PBM

Ten TERs from different origins were prepared according to Section 2.2.1. The female SD rats were randomly assigned to sham group, positive group and 10 TER drug groups, 12 rats in each group. Experimental methods and periods of each group were the same as the Section 2.2.2.

2.2.4. HPLC conditions

The chromatographic fingerprints were performed using Spurisil C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was composed of solvent A (acetonitrile) and solvent B (water solution) with a linear gradient as follows: 0–30 min: 30% A, 30–65 min: 60% A. The samples were monitored at the wavelength of 280 nm with a flow rate of 1.0 mL/min, column temperature of 30°C and injection volume of 20 μL.

2.2.5. Preparation of reference solution

The mixed reference solution containing adenosine, calycosin glycoside, ononin, calycosin and formononetin was prepared. Each reference substance was accurately weighed 0.001 g. All of the references were dissolved into 10 mL of methanol and then filtered through a 0.22 μm membrane to obtain the mixed reference solution.

2.2.6. Preparation of sample solution

Ten TERs from different origins were accurately weighed 0.50 g and dissolved in 10 mL methanol, then filtered through a 0.22 μm membrane to obtain the sample solutions.

3. Results

3.1. Pharmacodynamics experiments

3.1.1. PBM-enhancement effects of extracts from different polar parts of Radix Hedysari

As shown in Table 2, the results indicated a significant improvement in BMD and biomechanical parameters when comparing XLGB group, TER and HPS groups with sham group (p < 0.05). The serum biochemical parameters, AKP, TRACP, Ca, and P, changed significantly in TER and HPS groups compared with sham group (p < 0.05) and were comparable to XLGB group. The other three groups were not significantly changed. For the HPS group, a favorable result exhibited but not as better as the TER group. Thus in this research, the TER group was selected to explore the bioactive components of Radix Hedysari on the effect of improving PBM.

3.1.2. PBM-enhancement effects of 10 TER samples from different origins

As shown in Table 3, most of the parameters changed significantly in 10 TER groups compared with the sham group (p < 0.05), some of which extremely changed (p < 0.01), indicating that the modeling was successfully established and all of 10 TER samples showed a favorable effect on increasing PBM of rats.

3.2. HPLC fingerprints

3.2.1. Identification of compounds

The mixed reference solution containing adenosine, Calycosin-7-glucoside, ononin, calycosin, formononetin and 10 TER samples were operated in accordance with the steps in Sections 2.2.5 and 2.2.6, respectively. A total of 21 components of TER were obtained. By comparing the retention time and absorption wavelength...
between the TER samples and reference solution, the components of adenosine, Calycosin-7-glucoside, ononin, calycosin, and for- 
omonetin were unambiguously identified. The peak areas of 21 components of TER were recorded and analyzed using statistical 
methods. The HPLC-DAD fingerprints of TERs from 10 different 
sources and references are shown in Figs. 1 and 2, and chemical 
structures of five components are shown in Fig. 3.

3.2.2. Method validation

All of the samples were prepared in accordance with the 
method of Section 2.2.6. The precision of instruments was con-

Table 3

| Group | BMD (g·cm⁻²) | BMD of 1.5-month BMD-R | BMD of 2-month BMD-R | BMD of 3-month BMD-R | RSD | RSD% |
|-------|--------------|-------------------------|----------------------|----------------------|-----|------|
| Sham  | 0.138 ± 0.004 | 0.149 ± 0.009           | 0.153 ± 0.005        | 0.153 ± 0.003        | 0.08 | 5.75 |
| XGB   | 0.144 ± 0.005 | 0.150 ± 0.003           | 0.153 ± 0.005        | 0.153 ± 0.003        | 0.07 | 4.71 |
|       | 0.149 ± 0.009 | 0.153 ± 0.005           | 0.153 ± 0.005        | 0.153 ± 0.003        | 0.06 | 3.51 |
|       | 0.149 ± 0.002 | 0.153 ± 0.005           | 0.153 ± 0.005        | 0.153 ± 0.003        | 0.06 | 3.51 |

3.3. Statistical methods

3.3.1. Gray relational analysis

Gray relational analysis (GRA) between the common peak areas of 10 TERs fingerprints (Table 4) and all of the pharmacodynamic parameters (Table 3) was carried out by transforming the original data and calculating the correlation degree. The results are shown in Table 5. Ten TERs were divided into the high relational region (with correlation coefficient > 0.8), moderate relational region (0.7–0.8) and low relational region (< 0.7) according to the correlation coefficient. Considering all of pharmacodynamic factors, the common peaks of 3, 5, 6, 7, 8, 9, 10, and 12 had close correlation with the effect, of which peaks of 7, 8, 9, and 3 showed especially significant contribution to the effect and peaks of 7 and 9 were identified as ononin and iso-

3.3.2. Factor analysis and hierarchical clustering analysis

The factor scores of 10 TERs were calculated using SPSS 19.0. The original 10 parameters were simplified and integrated into 3 comprehensive factors (F). Each comprehensive factor could interpret the original information for 34% (a₁), 26% (a₂), and 16% (a₃), respectively. The cumulative contribution rate was 76%, which was in accordance with the requirement of more than 75%. The comprehensive score of 10 TERs was calculated as follows: $E = a₁F₁ + a₂F₂ + a₃F₃$ (E: comprehensive score, a₁~a₃: the contribution rate of each comprehensive factor, F₁~F₃: factor score of each comprehensive factor) [22] and is shown in Table 6. Rankings of 10 TERs were as follows on a descending order based on comprehensive scores: 4 > 3 > 7 > 8 > 1 > 5 > 10 > 9 > 6 > 2. In addition, hierarchical cluster analysis was carried out by using successive polymerizing. Squared euclidean distance was used to measure correlation degree between the 10 TERs while the class
The average relative retention time and peak area of common peaks of 10 TERs.

| Peak no. | \( t_R \) (min) | Average relative peak area |
|---------|-----------------|---------------------------|
| 1       | 7.497           | 373670                    |
| 2       | 9.618           | 307589                    |
| 3       | 13.971          | 111135                    |
| 4       | 18.797          | 95157                     |
| 5       | 19.867          | 52043                     |
| 6       | 22.644          | 143632                    |
| 7       | 31.166          | 3225806                   |
| 8       | 34.419          | 154142                    |
| 9       | 35.450          | 191028                    |
| 10      | 36.389          | 1768080                   |
| 11      | 37.563          | 113391                    |
| 12      | 39.323          | 167234                    |
| 13      | 46.754          | 439623                    |
| 14      | 50.099          | 657044                    |
| 15      | 51.289          | 427064                    |
| 16      | 52.479          | 3297751                   |
| 17      | 55.878          | 176363                    |
| 18      | 57.033          | 1757055                   |
| 19      | 59.805          | 221785                    |
| 20      | 61.350          | 308326                    |
| 21      | 67.374          | 335803                    |

average chain method was used to measure the correlation degree among the various classes. The results are shown in Fig. 4 and 10 TERs were divided into three clusters when the rescaled distance cluster was 15. Cluster 1 consisted of samples 3, 4, 7, and 8. Cluster 2 included samples 1, 5, 6, 9, and 10. Sample 2 represented cluster 3 alone. In conclusion, hierarchical cluster analysis provides a qualitative comparison of samples and the result was consistent with factor analysis.

4. Discussion
4.1. Pharmacodynamics experiment

The overall bone properties are determined based on the microstructure, geometry and material mechanics performance [23]. Bone voluntarily modifies its material composition and structure to accommodate loads by adaptive modeling and remodeling [24]. If the bone mass increases, the flexibility and toughness of bone can also be improved while the risk of fracture is reduced in terms of bone biomechanical properties. The mechanical indicators of peak load, elastic modulus and yield strength are mainly...
that the bone withstands before fracture [20]. Modulus of measuring osteoporosis. Peak load re considered to investigate the curative effect which are criteria for.

The dendrogram of cluster analysis of 10 TERs from different origins. The

Fig. 4. Each factor score and comprehensive scores of 10 TERs.

Table 6

Gray relational grades (GRG) between the 21 common peaks of 10 TERs and each indicator (sorted in descending order).

| TER no. | F1 (34%) | F2 (26%) | F3 (16%) | E   |
|---------|----------|----------|----------|-----|
| 1       | -0.324   | 0.339    | 1.195    | -0.007 |
| 2       | -1.357   | 0.032    | -0.545   | -0.538 |
| 3       | 0.4276   | 0.091    | 0.7385   | 0.4437 |
| 4       | 1.4223   | 0.5299   | 1.4243   | 0.8492 |
| 5       | -0.8136  | 0.8737   | -0.3028  | -0.0978 |
| 6       | -0.5973  | -1.3363  | 0.3326   | -0.4973 |
| 7       | 0.3656   | 1.6051   | -1.5489  | 0.2938 |
| 8       | 1.5368   | -0.6123  | -0.7779  | 0.2486 |
| 9       | 0.4170   | -1.6106  | -1.1314  | -0.4580 |
| 10      | -1.0808  | 0.1649   | 0.5529   | -0.2361 |

considered to investigate the curative effect which are criteria for measuring osteoporosis. Peak load reflects the maximum force that the bone withstands before fracture [20]. Modulus of elasticity is used to measure the abilities of bone. Yield strength is a measure of the elastic limit of bone and is determined as the point in which the slope of the load-deformation curve deviates from a straight line [25]. The above three indicators detected in the paper showed significant difference between the TER group, positive group and sham group (p < 0.05, p < 0.01), which fully confirmed that the TER group could increase PBM and further improve the condition of osteoporosis. In addition, the universal standard for diagnosis of osteoporosis is the dual energy X-ray absorption method for the determination of bone mineral density. It plays a significant role in the osteoporosis diagnosis because of its direct detection of bone mass [26]. In this article, BMD-B of all the rats was measured after dosing 1.5 month in order to monitor BMD in the mid-stage of experiment, which showed a certain trend on improving PBM. After three months, BMD-B and BMD-R of rats showed significant differences between TER group, positive group and sham group (p < 0.05), indicating a remarkable effect of TER group on improving bone mineral density, which is of a great reference value for increasing PBM. Besides, although the serum biochemical indicator is not a substitute for BMD instrument, it can indicate the state of bone turnover quickly and sensitively, which plays a significant role in real-time monitoring for drug efficacy and evaluation for bone quality. Serum TRACP is derived from bone, prostate, red blood cells and platelets, secreted by the osteoclasts in bone tissue and then releases into the blood stream. It is characteristic of the bone resorption enzyme, which has been used since 1982 as a diagnosis index for bone resorption. Serum AKP mainly comes from bone and liver, which accounts for about half of total bone type. The bone AKP is an intracellular enzyme, releasing inorganic phosphorus by hydrolyzing phospholipids on the cell membrane of ossification to get a higher concentration, promoting matrix mineralization and thus increasing the bone mineral content. This index is mainly used to express the activity of osteoblast. Blood Ca and blood P are abundant in bone tissue as bone minerals. If the resorption of bone increases, Ca and P would release into the blood, reflecting the imbalance of bone resorption and bone formation. All of the serum indicators were detected and the TER group showed significant

Table 5

Gray relational grades (GRG) between the 21 common peaks of 10 TERs and each indicator (sorted in descending order).
differences compared with sham group, indicating the effect of increasing PBM on the field of biochemistry. In conclusion, we considered the bone mineral density, bone biomechanical parameters and serum index comprehensively with the results were reasonable and reliable.

4.2. Statistical methods

GRA is a quantitative method based on the gray system theory, which describes the degree of correlation between the objects and factors with relative coefficient. The greater the relative coefficient is, the closer the similarity would be [27]. GRA is conducted to rank the influence of compared series in an imaging gray space, using the relative distance between them without making prior assumption about the distribution type [28]. The correlation and rank of relative coefficient are obtained by conversing original data, getting the absolute difference sequence, then determining correlation coefficient to confirm the relevancy degree. In the previous articles, only singleness indicator was selected on the research of spectrum-effect relationship, the result of which showed characteristics of singleness, randomicity and incomprehensiveness. In this article, considering all the effect indexes creatively, 21 common peaks were divided into three sections based on the relative coefficient ( > 0.8, 0.7–0.8, < 0.7). Five chemical compounds were identified in this paper, of which glycosides and calycosin played an extremely significant role in improving PBM, and adenosine, calycosin and fermlononetin also had certain contribution.

In addition, considering all of the indicators, 10 TERs were classified into different groups using the factor analysis and hierarchical cluster analysis. Factor analysis is to use a few comprehensive indexes to describe the link between a large number of indicators or factors by adopting the idea of dimension reduction, using a few common factors instead of many of the original indicators to make the problem simplified and easy to calculate. In the article, three comprehensive factors were extracted, the cumulative contribution rate of which reached 76%. Ten TERs were sorted based on the comprehensive factor scores, which were obtained by weighing each common factor score. In the hierarchical clustering analysis, 10 TER samples were divided into three categories, the result of which was consistent with that of the factor analysis. The two methods are complementary and authenticated with each other, which offers a reliable method to screen the optimal producing area for Radix Hedysari.

4.3. HPLC chromatographic conditions for optimization

A series of conditions of chromatographic column, mobile phase, column temperature and flow rate were optimized. Different types of chromatographic columns from different manufacturers were tested to choose the optimal one. The mobile phases of methanol-water and acetonitrile-water with different ratios were explored, in which the acetonitrile-water with strong ability was selected for gradient elution ultimately. The column temperature was adjusted within the range from 20 °C to 45 °C. The flow rate was also optimized from 0.5 mL/min to 2.0 mL/min.

5. Conclusion

In this article, the extracts from different polar parts of Radix Hedysari are prepared for preliminary determination of bioactive compounds, in which TER is found to be the most effective extract. Then the spectrum-effect relationship is established to accurately screen the bioactive compounds for increasing PBM of rats, which has been identified by HPLC-DAD. In addition, the best producing area for Radix Hedysari is confirmed by using factor analysis and clustering analysis. This work provides an efficient and comprehensive approach to evaluate Radix Hedysari and further develop its medical value.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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