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Original Article

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.

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 maturing 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

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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 absorptiometry (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).

Table 1
Sources of *Radix Hedysari.*

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	Ganquan Town of WuDu
10	Lichuan Town of TanChang

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.

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], XIANLINGUBAO (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 absorptiometry (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 Spursil C_{18} 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

Table 2
Effect of different extracts of *Radix Hedysari* on PBM of rats (mean ± SD, $n = 12$).

Group	BMD (g/cm^2)			Biomechanical indexes			Serum parameters			
	BMD-B of 1.5 month	BMD-B of 3 month	BMD-R	Maximum load (N)	Modulus of elasticity (mPa)	Yield strength (mPa)	TRACP (U/L)	AKP (U/L)	Ca (mmol/L)	P (mmol/L)
Sham	0.141 ± 0.007	0.144 ± 0.009	0.11 ± 0.003	120.23 ± 7.39	1532.87 ± 186.5	120.11 ± 6.90	9.3 ± 0.012	60.3 ± 0.031	2.6 ± 0.012	4.3 ± 0.017
XLGB	0.145 ± 0.003*	0.155 ± 0.005**	0.115 ± 0.004*	133.34 ± 10.88**	2298.23 ± 192.06**	124.45 ± 9.29*	8.1 ± 0.009**	70.5 ± 0.022**	1.8 ± 0.011*	3.6 ± 0.019*
TER	0.144 ± 0.014*	0.158 ± 0.006**	0.114 ± 0.007*	127.03 ± 9.31*	1934.87 ± 238.4*	124.78 ± 5.89*	8.4 ± 0.021**	68.5 ± 0.051**	2.0 ± 0.011*	3.5 ± 0.016*
HPS	0.142 ± 0.011	0.153 ± 0.001*	0.112 ± 0.012	123.16 ± 13.28	1726.49 ± 290.12*	122.57 ± 11.39*	8.6 ± 0.031*	64.5 ± 0.029*	2.4 ± 0.020	3.9 ± 0.023*
PER	0.136 ± 0.015	0.148 ± 0.008*	0.106 ± 0.004	117.86 ± 15.39	1590.33 ± 178.23	120.41 ± 12.44	9.2 ± 0.016	61.1 ± 0.034	2.5 ± 0.016	4.5 ± 0.019
EAR	0.134 ± 0.012	0.139 ± 0.003	0.104 ± 0.015	116.60 ± 13.90	1690.22 ± 249.25*	118.20 ± 8.92	9.3 ± 0.024	60.8 ± 0.055	3.0 ± 0.008	4.2 ± 0.011
EER	0.135 ± 0.01	0.151 ± 0.011*	0.107 ± 0.009	121.14 ± 18.65	1522.02 ± 268.13	118.77 ± 10.25	8.7 ± 0.015	59.4 ± 0.034	2.6 ± 0.018	4.6 ± 0.022

TER represents total extract, HPS represents polysaccharide, PER represents petroleum ether extract, EAR represents ethyl acetate extract, EER represents ethanol extract of *Radix Hedysari*, respectively.

** $p < 0.01$, * $p < 0.05$, compared with sham group.

Table 3
Effect of 10 TERs from different sources on increasing PBM of rats (mean ± SD, $n = 12$).

Groups and TERs	BMD (g/cm^2)			Biomechanical indexes			Serum parameters			
	BMD-B of 1.5 month	BMD-B of 3 month	BMD-R	Maximum load (N)	Modulus of elasticity (mPa)	Yield strength (mPa)	TRACP (U/L)	AKP (U/L)	Ca (mmol/L)	P (mmol/L)
Sham	0.138 ± 0.004	0.141 ± 0.003	0.108 ± 0.014	117.55 ± 6.18	1444.39 ± 182.59	118.18 ± 5.53	9.63 ± 0.024	13.18 ± 0.44	2.44 ± 0.54	4.12 ± 0.67
XLGB	0.146 ± 0.005**	0.150 ± 0.003*	0.119 ± 0.013**	127.56 ± 19.53*	2052.50 ± 135.58**	120.02 ± 9.20	8.37 ± 0.015**	19.22 ± 0.28**	1.98 ± 0.45*	3.52 ± 0.22*
1	0.141 ± 0.007	0.153 ± 0.003**	0.114 ± 0.016*	119.18 ± 6.81*	1839.08 ± 84.98*	116.73 ± 8.33	8.62 ± 0.035**	19.43 ± 0.27**	2.06 ± 0.47*	3.22 ± 0.34*
2	0.143 ± 0.009*	0.153 ± 0.005**	0.117 ± 0.011**	114.03 ± 5.15	1592.19 ± 12.71	109.45 ± 1.34	8.49 ± 0.055**	26.18 ± 1.00**	2.03 ± 0.46*	3.87 ± 0.12*
3	0.149 ± 0.003**	0.153 ± 0.007**	0.120 ± 0.019**	134.17 ± 26.95**	1795.55 ± 488.38*	126.33 ± 24.25*	9.01 ± 0.028*	19.62 ± 0.21**	1.92 ± 0.43*	3.43 ± 0.16*
2	0.143 ± 0.009*	0.153 ± 0.005**	0.117 ± 0.011**	114.03 ± 5.15	1592.19 ± 12.71	109.45 ± 1.34	8.49 ± 0.055**	26.18 ± 1.00**	2.03 ± 0.46*	3.87 ± 0.12*
4	0.147 ± 0.009**	0.156 ± 0.002**	0.125 ± 0.009**	146.27 ± 8.87**	1985.41 ± 307.09*	132.33 ± 13.80*	8.92 ± 0.037*	21.33 ± 0.43**	1.89 ± 0.39**	3.79 ± 0.23*
5	0.142 ± 0.005*	0.149 ± 0.005*	0.115 ± 0.004*	124.43 ± 16.17*	1880.49 ± 393.24*	130.75 ± 3.75*	8.98 ± 0.045*	18.79 ± 0.57*	1.94 ± 0.38*	3.10 ± 0.61**
6	0.140 ± 0.002	0.149 ± 0.004*	0.115 ± 0.014*	126.77 ± 9.60*	1567.66 ± 280.52	129.10 ± 7.23*	8.44 ± 0.012**	14.59 ± 0.26*	1.93 ± 0.37*	3.04 ± 0.33**
7	0.142 ± 0.002*	0.156 ± 0.005**	0.121 ± 0.009**	123.57 ± 25.17*	1726.30 ± 267.45*	123.27 ± 7.66	9.34 ± 0.021	14.46 ± 0.18*	1.83 ± 0.35**	3.81 ± 0.18*
8	0.142 ± 0.007*	0.151 ± 0.004*	0.117 ± 0.011**	142.50 ± 18.46**	2032.40 ± 136.71**	119.10 ± 10.45	9.21 ± 0.033	15.48 ± 0.21*	1.79 ± 0.39**	3.28 ± 0.29*
9	0.136 ± 0.006	0.153 ± 0.007**	0.117 ± 0.012**	133.15 ± 10.78**	1921.45 ± 168.48*	128.95 ± 19.45*	8.49 ± 0.018**	11.66 ± 0.19	1.74 ± 0.31**	3.19 ± 0.27*
10	0.139 ± 0.006	0.152 ± 0.002**	0.118 ± 0.012**	110.75 ± 11.45	1651.65 ± 158.96	132.12 ± 9.86*	9.03 ± 0.031*	14.16 ± 0.29*	1.71 ± 0.27**	3.55 ± 0.37*

**$p < 0.01$, *$p < 0.05$, compared with sham group.

between the TER samples and reference solution, the components of adenosine, Calycosin-7-glucoside, ononin, calycosin, and formononetin were unambiguously identified. The peak areas of 21 components of TER were recorded and analyzed using statistic 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 confirmed by repetitively injecting 20 μL of reference solution of formononetin linarin for five times. The TER sample solution was injected repeatedly at 0, 3, 6, 9, 10 and 12 h to explore stability with an injection of 20 μL. The repeatability of the method was validated through six parallel experiments. All of the relative retention times and relative peak areas of chromatographic peaks were recorded, the relative standard deviation (RSD) values of which were all below 3.0%, indicating that the instrument had a good precision, sample solution was stable for at least 12 h and the method was also repeated well. The recovery experiment was accomplished by adding certain amounts of reference solutions (adenosine, Calycosin-7-glucoside, ononin, calycosin, and formononetin) into the TER sample and repeated six times. The average recovery rate was 98.18%, 97.08%, 99.23%, 103.33%, and 97.09% with RSD of 1.99%, 2.32%, 1.81%, 1.73%, and 2.04%, respectively.

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 isoflavone. In moderate relational region, the correlation coefficient of peaks of 1, 2, 4, 11, 13, 14, 16, 17, 18, 19, and 21 were between 0.7 and 0.8, of which 1, 6, and 13 were identified as adenosine, campanulin and formononetin. Each peak in this region has certain contribution to the efficacy. There are common peaks of 15 and 20 mainly in low correlation region, which had little contribution to the efficacy.

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 interpretate the original information for 34% (a_1), 26% (a_2), and 16% (a_3), 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: $E = a_1F_1 + a_2F_2 + a_3F_3$ (E: comprehensive score, $a_1 \sim a_3$: the contribution rate of each comprehensive factor, $F_1 \sim F_3$: 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

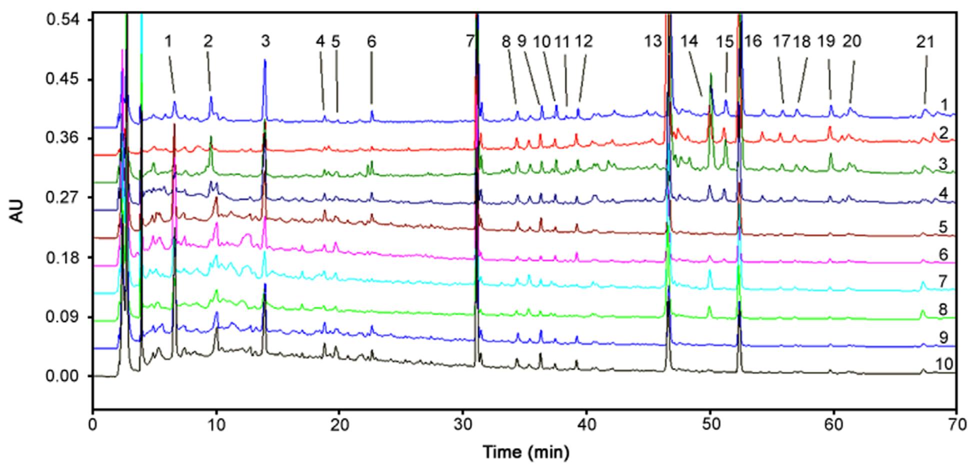

Fig. 1. HPLC-DAD fingerprints of TERs from 10 different sources. Peak identifications: 1. adenosine; 6. calycosin-7-glucoside; 7. ononin; 9. calycosin; 13. formononetin.

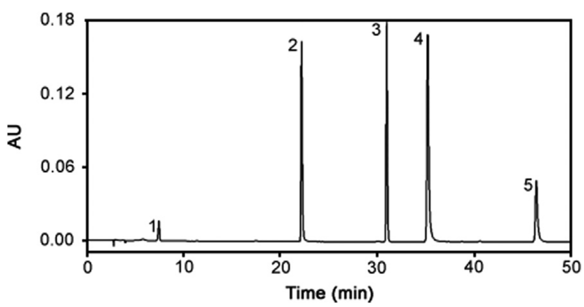

Fig. 2. HPLC fingerprints of references (280 nm). Peak identifications: 1. adenosine; 2. calycosin-7-glucoside; 3. ononin; 4. calycosin; 5. formononetin.

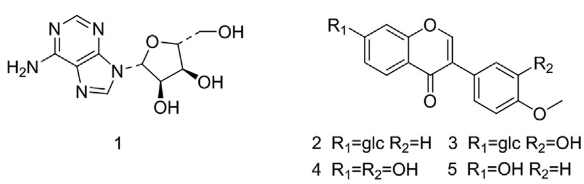

Fig. 3. Chemical structures of five components. Identifications: 1. adenosine; 2. Calycosin-7-glucoside; 3. ononin; 4. calycosin; 5. formononetin.

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 conclude, 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

Table 4
The average relative retention time and peak area of common peaks of 10 TERs.

Peak no.	t_R (min)	Average relative peak area									
		1	2	3	4	5	6	7	8	9	10
1	7.497	373670	139241	264211	451159	1815778	1639354	855715	632750	1527688	2424186
2	9.618	307589	129703	1118470	201462	580226	835359	115281	460292	466222	578057
3	13.971	1111335	545334	1176260	1293862	945582	608290	460409	609337	926858	1103359
4	18.797	95157	43036	56693	107603	238634	147143	388243	228134	182831	211116
5	19.867	52043	13757	112722	90719	155699	242954	148393	123231	114439	249267
6	22.644	143632	47469	192445	101085	175981	109805	30567	39433	133755	123631
7	31.166	3225806	2625018	3271197	2520147	2398170	2356950	1411869	956191	2329398	2769968
8	34.419	154142	161867	284113	142900	199623	54022	178500	113572	166257	141160
9	35.450	116611	172428	186864	158863	100590	70257	234315	157724	84443	107319
10	36.389	191028	192043	350465	135132	197338	74731	114696	72513	180124	232992
11	37.563	176808	113391	171311	104888	60321	73028	26817	25882	50842	68591
12	39.323	167234	208297	191492	112961	125529	127177	54465	30146	92419	139386
13	46.754	4108663	439623	4817087	2724719	1220437	1432728	2891621	1856606	1067069	1497406
14	50.099	697044	799765	2145133	357930	45242	139946	426984	250598	38033	70288
15	51.289	427064	324567	757398	312661	20351	42723	15961	20221	20112	16572
16	52.479	3297751	4395627	3122347	1840209	964846	693914	1598948	1004739	816068	1176831
17	55.878	176363	175705	156814	98299	27033	36834	64133	42411	19213	27713
18	57.033	178985	160123	131551	102267	26087	26233	75239	52828	21502	28899
19	59.805	221785	349607	391301	106724	54956	67160	62581	43300	40601	65168
20	61.350	308326	206057	346037	114341	32418	30094	77836	30633	25890	31898
21	67.374	335803	167503	304526	136240	84735	100026	239739	257645	80860	115638

Table 5
Gray relational grades (GRG) between the 21 common peaks of 10 TERs and each indicator (sorted in descending order).

BMD (g/cm^2)						Biomechanical indexes						Serum parameters							
BMD-B of 1.5 month		BMD-B of 3 month		BMD-R		Maximum load (N)		Modulus of elasticity (mPa)		Yield strength (mPa)		TRACP (U/L)		AKP (U/L)		Ca (mmol/L)		P (mmol/L)	
7[a]	0.9035	7	0.9021	7	0.8999	7	0.8777	8	0.8812	7	0.8985	7	0.8959	9	0.8617	7	0.9021	7	0.8893
8	0.8886	8	0.8925	8	0.8882	8	0.8761	7	0.8779	8	0.8821	8	0.8902	7	0.8565	8	0.8825	8	0.8808
9	0.8571	3	0.8583	9	0.8579	9	0.8683	3	0.8699	3	0.8803	9	0.8577	8	0.8544	12	0.8550	9	0.8764
3	0.8554	9	0.8563	3	0.8559	3	0.8553	9	0.8605	10	0.8485	3	0.8566	3	0.8505	3	0.8525	3	0.8485
12	0.8471	12	0.8466	12	0.8406	12	0.8221	10	0.8384	12	0.8414	12	0.8447	11	0.8337	9	0.8514	10	0.8362
10	0.8418	10	0.8464	10	0.8391	10	0.8219	12	0.8228	9	0.8358	10	0.8409	12	0.8336	10	0.8499	12	0.8359
5	0.8157	5	0.8201	5	0.8168	6	0.8056	6	0.8124	6	0.8320	5	0.8241	10	0.8176	5	0.8172	5	0.8200
6	0.8153	6	0.8201	6	0.8135	21	0.7994	4	0.8116	5	0.8148	6	0.8170	16	0.8165	6	0.8158	11	0.8046
21	0.7977	21	0.8006	21	0.7961	5	0.7983	21	0.8044	4	0.8014	21	0.8055	13	0.8120	11	0.7980	6	0.7969
2	0.7924	4	0.7992	4	0.7958	4	0.7945	5	0.8028	2	0.7995	4	0.8051	18	0.7987	21	0.7957	21	0.7961
4	0.7915	2	0.7935	2	0.7919	11	0.7894	11	0.7910	11	0.7799	2	0.7953	21	0.7886	2	0.7865	13	0.7949
11	0.7906	11	0.7909	11	0.7897	13	0.7833	13	0.7849	21	0.7798	11	0.7844	17	0.7859	13	0.7836	4	0.7939
13	0.7820	13	0.7847	13	0.7839	2	0.7786	2	0.7797	13	0.7772	13	0.7825	6	0.7743	16	0.7831	2	0.7826
16	0.7725	16	0.7738	16	0.7680	16	0.7611	16	0.7665	16	0.7628	16	0.7686	5	0.7642	4	0.7790	16	0.7711
18	0.7467	18	0.7467	18	0.7457	18	0.7458	18	0.7468	18	0.7440	18	0.7399	14	0.7521	18	0.7535	18	0.7532
17	0.7299	17	0.7305	17	0.7296	17	0.7299	17	0.7318	1	0.7393	17	0.7242	2	0.7424	17	0.7360	17	0.7379
1	0.7215	1	0.7270	1	0.7233	1	0.7192	1	0.7237	17	0.7275	1	0.7238	19	0.7410	1	0.7220	1	0.7124
14	0.7069	14	0.7097	14	0.7038	14	0.6937	14	0.7025	14	0.6997	14	0.7033	4	0.7363	14	0.7191	14	0.7084
19	0.7039	19	0.7067	19	0.7012	19	0.6933	19	0.7011	19	0.6945	19	0.7019	1	0.7328	19	0.7137	19	0.7058
20	0.6931	20	0.6957	20	0.6902	20	0.6809	20	0.6874	20	0.6839	20	0.6909	20	0.6897	20	0.6725	20	0.6954
15	0.6343	15	0.6367	15	0.6349	15	0.6313	15	0.6352	15	0.6290	15	0.6311	15	0.6863	15	0.6411	15	0.6448

[a] The labels of 21 common peaks.

Table 6
Each factor score and comprehensive scores of 10 TERs.

TER no.	F_1 (34%)	F_2 (26%)	F_3 (16%)	E
1	− 0.3240	− 0.3398	1.1953	− 0.0073
2	− 1.3537	0.0322	− 0.5435	− 0.5388
3	0.4276	0.6930	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	− 0.7170	0.2486
9	0.4170	− 1.6106	− 1.1314	− 0.4580
10	− 1.0808	0.1649	0.5529	− 0.2361

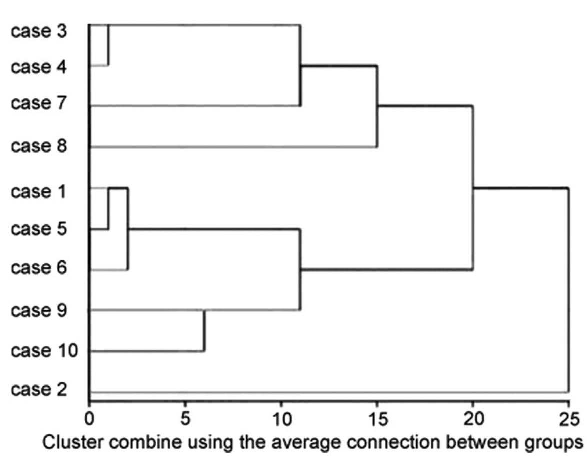

Fig. 4. The dendrogram of cluster analysis of 10 TERs from different origins. The clustering method was nearest neighbor and the distance calculating method was euclidean distance.

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 increasing 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 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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