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ORIGINAL ARTICLE

Spectrum–Effect Relationships between Fingerprints of Radix *Polygoni multiflori-Achyranthes bidentate* and Antiosteoporosis Effect Based on Different Extraction Solvents

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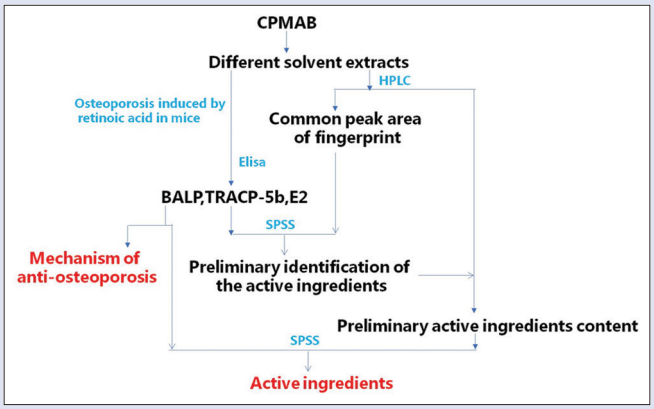
ABSTRACT

Background: *Polygonum multiflorum* and *Achyranthes bidentate* are traditional Chinese herbs, which show significant antiosteoporosis (OP) effect. These two herbs are commonly used as a combination in traditional Chinese medicine. **Materials and Methods:** In this study, the left humerus section of the mice were stained with hematoxylin and eosin staining to establish the spectrum-effect relationships between the high-performance liquid chromatography fingerprint of different solvent extracts and the anti-OP effect. Finally, we analyzed the correlation between the content of main compounds of different solvent extracts and the anti-OP effect. **Results:** The compatibility had obvious therapeutic effect on OP induced by retinoic acid. The results of pharmacodynamic analysis showed that the extract had a certain effect on the bone-specific alkaline phosphatase, the level of tartrate-resistant acid phosphatase-5b (TRACP-5b), and estrogen 2 and had a great influence on TRACP-5b especially. The results of spectrum-effect relationships and correlation analysis showed that the level of β-ecdysterone and tetrahydroxystilbene is negatively correlated with bone resorption index and TRACP-5b. The higher the content of the two compounds in the combination, the better the effect of treating mouse OP induced by retinoic acid. **Conclusion:** It is suggested that the compatibility of *P. multiflorum* and *A. bidentata* can be used to treat OP, and their mechanism of action is through reducing osteoclastic effect. β-ecdysterone and tetrahydroxystilbene may be the active components of combination of *Polygoni multiflori-A. bidentate*.

Key words: Active ingredient, antiosteoporosis, enzyme-linked immunosorbent assay, fingerprint, spectrum-effect relationships

SUMMARY

- *Polygonum multiflorum* and *Achyranthes bidentate* are traditional Chinese herbs. They demonstrate significant antiosteoporosis (OP) effect. In this experiment, the spectrum–effect relationships between the high-performance liquid chromatography fingerprint of different solvent extracts, and the anti-OP effect was established and the correlation between the content of main compounds of different solvent extracts, and the anti-OP effect was analyzed. The higher the content of the β-ecdysterone and tetrahydroxystilbene in the combination drug, the better the anti-OP effect. It is suggested that the compatibility of *P. multiflorum* and *A. bidentata* can be used to treat OP, and its mechanism may be to reduce osteoclastic effect. β-Ecdysterone and tetrahydroxystilbene may be the active components of compatibility of *Polygoni multiflori-A. bidentate*.



Abbreviations used: OP: Osteoporosis; CPMAB: Compatibility of *Polygoni multiflori-Achyranthes bidentate;* TRACP-5b: Tartrate-resistant acid phosphatase 5b; BALP: Bone specific alkaline phosphatase; E2: Estradiol 2; HPLC: High performance liquid chromatography; H and E: Hematoxylin and Eosin stain; KM: KunMing spices; OD: Optical density.

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INTRODUCTION

Osteoporosis (OP), one of the three kinds of metabolic osteopathies (the other two being osteomalacia and osteopenia), is characterized by a reduced bone mass and increased destruction of bone microstructures. A person affected with this condition shows decreased bone strength, increased fragility, and increased chances of fracture.[1] OP can be categorized into primary, secondary, and idiopathic OP. Primary OP can be further classified into postmenopausal and senile OP.[2] Worldwide, more than 200 million people suffer from various types

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of OP, of which about 1.6 million experience fracture. By 2050, the estimated number of hip fractures worldwide is approximately 6.26 million.[3] Patients with OP typically exhibit a common feature: the imbalance between bone formation and bone resorption. In OP, the individuals exhibit normal rate of bone formation but with an increased bone resorption rate.[4] Therefore, serum bone resorption indexes such as C-terminal telopeptide of type I collagen (CTX-1) tartrate-resistant acid phosphatase 5b (TRACP-5b) and bone formation index such as bone-specific alkaline phosphatase (BALP) are often important indicators for the detection of OP.[5] In addition, as a first-line drug for the prevention and treatment of postmenopausal OP, 17β-estradiol (E2) is also an important index in the maintenance of balance between bone formation and bone resorption.[6] Recent studies show that retinoic acid can duplicate OP model in mice.[7,8]

The combination of *Polygoni multiflori-Achyranthes bidentate* (CPMAB) is a traditional Chinese medicine and is commonly used in clinical setting. It originated from the Pill of Radix *P. multiflori* in *Taiping Shenghui Prescription*, which is a traditional tonic. *P. multiflori* is the dried root of *Polygonum multiflorum*. It tonifies the liver and kidney, nourishes blood, prevents graying of hair, and strengthens tendons and bones.[9] *Achyranthes bidentata* is the dried root of *A. bidentata*.[9] Radix *P. multiflori* combined with *A. bidentata* greatly enhance the aforementioned effects.[10] Modern studies have shown that Radix *P. multiflori* and Radix *A. bidentata* both have significant anti-OP effects.[7,8,11] β-Ecdysterone is the main active ingredient of *A. bidentate*.[12,13] Tetrahydroxystilbene glucoside, emodin, and emodin methyl ether are the main active ingredient of *P. multiflorum* Thunb.[14,15] Recent studies have shown that β-ecdysterone, tetrahydroxystilbene glucoside, emodin, and emodin methyl ether all have good anti-OP effects.[16-21] Literature describes different methods of extraction using different extraction solvents.[22-25]

High-performance liquid chromatographic (HPLC) fingerprints of different extraction solvents were established. The anti-OP activity of the CPMAB was confirmed by pharmacological experiments. We induced OP by retinoic acid. The primary goal of this study was to obtain the best-extracting solvent, the effective components of CPMAB against OP, and to identify the active ingredients of CPMAB.

MATERIALS AND METHODS

Materials and reagents

Radix *P. multiflori* and *A. bidentata* were purchased in Bozhou Medicinal Material Market, Anhui Province, China, and they were identified as the roots of *P. multiflorum* Thunb. and *A. bidentata* Bl. by Professor Zhai Yanjun of Liaoning University of Traditional Chinese Medicine. Experimental research on plants (either cultivated or wild), including the collection of plant material, complied with the *IUCN Policy Statement on Research Involving Species at Risk of Extinction* and the *Convention on the Trade in Endangered Species of Wild Fauna and Flora*. Reference substances of β-ecdysterone, tetrahydroxystilbene glucoside, emodin, and emodin methyl ether were purchased from China Institute of Food and Drug Verification with purity of more than 98% (Beijing, China).

HPLC-grade acetonitrile and methanol were purchased from Komeo Chemical Reagent Co., Ltd (Tianjin, China).

Hematoxylin and eosin stain (H and E) kits, BALP, TRACP-5b, and E2 were purchased from Shanghai Enzyme-Linked Biotechnology Co., Ltd. (Shanghai, China). Retinoic acid was purchased from Beijing Solebo Technology Co., Ltd. (Beijing, China).

Specific pathogen-free Kunming (KM) mice (half male and half female) (6–8 weeks old, weighing 18–22 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China). The animals were kept in an environment with constant temperature, humidity, and light conditions, with food and water provided *ad libitum*. All mice were acclimated in the laboratory for at least 1 week before the experiment. Before testing, the animals were fasted overnight with free drinking water.

All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals, and the study protocols were approved by the Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine (license: SYXK (辽) 2013-0009). Euthanasia of mice conforms to the group standard of Chinese Society of Experimental Animals (T/CALAS 31-2017).

Sample preparation

The powder of the same quality of Radix *P. multiflori* and *A. bidentata* was mixed and extracted five times with different solvents. After reflux extraction for 2 h, the solvent was filtered and dried. The residue was dissolved in methanol to obtain 0.1 g/mL solution, which was filtered with 0.22 μm microporous membrane for HPLC. The residues were dissolved in purified water for pharmacodynamics experiment.

The concentration of mixed standard solutions containing β-ecdysterone, tetrahydroxystilbene glucoside, emodin, and emodin methyl ether was 0.58, 1.18, 0.36, and 0.76 mg/mL by adding methanol.

Retinoic acid was precisely weighed and dissolved in saline to prepare a solution of 0.03 g/mL concentration.

Analysis of high-performance liquid chromatography fingerprints

High-performance liquid chromatography conditions

The samples were injected into a 1100 HPLC system (SHIMADZU, JAPAN) with SPD-10AVP UV detector. To correct the conditions of liquid chromatography, we investigated the absorption wavelength of the mobile phase. The chromatographic conditions were determined as follows: chromatographic column was Agilent Eclipse XDB-C_{18} (5 μm, 4.6 × 250 mm, USA), phase A was acetonitrile, and phase B was water. Table 1 shows the gradient elution procedure. The absorption wavelength was 250 nm, and the injection volume was 10 μL. The temperature of the column incubator was 30°C.

Validation of methodology

The relative retention time and peak area were 0.07% and 1.17% for β-ecdysterone, 0.05% and 1.19% for tetrahydroxystilbene glucoside, 0.18% and 2.47% for emodin, and 0.26% and 2.79% for emodin, respectively. The relative retention time and peak area were 0.04% and 2.47% for β-ecdysterone, 0.17% and 1.97% for tetrahydroxystilbene glucoside, 0.11% and 2.66% for emodin, and 0.21% and 2.17% for emodin, respectively. The relative retention time of the stability experiment was below 2.30%, and the peak areas were below 2.73%. The results showed that the method used for the HPLC fingerprint was valid and suitable.

Table 1: Gradient elution procedure

Time (min)	A (%)	B (%)
0-5	5	95
5-10	17	83
10-26	19	81
26-46	50	50
46-61	100	0
61-66	5	95

Calculation of relative retention time and relative chromatographic peak area

Peak 5 (X5) was designated as the reference peak. According to the ratio of retention time and area of each fingerprint peak to reference peak, the relative retention time and area of each fingerprint peak were calculated.

Antiosteoporosis experiment

KM mice (n = 80) were randomly divided into eight groups (n = 10 for each group, male and female half). One of the groups was "normal," the other seven groups were induced administered with retinoic acid through intragastric administration for 14 days. After 12 h of retinoic acid induction every day, the different solvent extracts (12 g/kg) of the study compounds were fed by gavage. At present, there is no report on the treatment of OP with the CPMAB, so they administered in a ratio of 50:50[24.26] Retinoic acid was dissolved in normal saline and administered at a dose of 105 mg/kg.[27]

Preparation of bone sections

All animals were anesthetized by the intraperitoneal administration of pentobarbital sodium (dose 50 mg/kg) for 15 min. Until the respiratory frequency of mice decreased, the main respiratory rule was abdominal respiration, the eyelid reflex disappeared, the corneal reflex was weak, and no harmful reflex was caused upon severe stimulation. The animals were sacrificed through cervical dislocation. Then, the tibia of the left hind leg was collected. According to the preparation method of paraffin section of bone tissue, the samples were stained with H and E kit. Then, the pathological changes were observed under light microscopy (E200, NIKON, JAPAN).

Enzyme-linked immunosorbent assay

Blood was collected from the orbital vein of the mice 24 h after the last administration. The cervical vertebra was dislocated after the mice were anesthetized with pentobarbital sodium (dose 50 mg/kg). The blood was allowed to stand at room temperature for 20 min and then centrifuged at 12,000 rpm for 20 min to separate the serum. According to the manufacturer's instructions, the optical density (OD) value of each sample was determined by enzyme-linked immunosorbent assay (ELISA) at 450 nm. The contents of BALP, TRACP-5b, and E2 in mice sera were detected. The results showed that there was a significant linear relationship between cytokine concentration and OD value. The cytokine concentration of each sample was determined according to the standard curve of cytokine and OD values. The data were analyzed to test the effect of drugs on OP. Data were presented as mean ± standard deviation (SD). Data were analyzed using SPSS 19.0 statistical package. Analysis of variance and eight sets of tests were designed based on complete randomization for single factor (least square difference method).

Analysis of spectrum-effect relationships

Multiple linear correlation analysis was conducted by SPSS 19.0 (Statistical Product and Service Solutions, IBM) software. The relative area of each peak ($X1$–$X10$) and the content of four active components (β-ecdysterone, tetrahydroxystilbene glucoside, emodin, and emodin methyl ether) in HPLC fingerprint were set as independent variables (X), and the content of anti-OP factor was set as independent variable (Y). According to the results of multiple linear correlation analysis, the equation was established. The chromatographic peaks (compounds) with significant anti-OP effect were screened out.

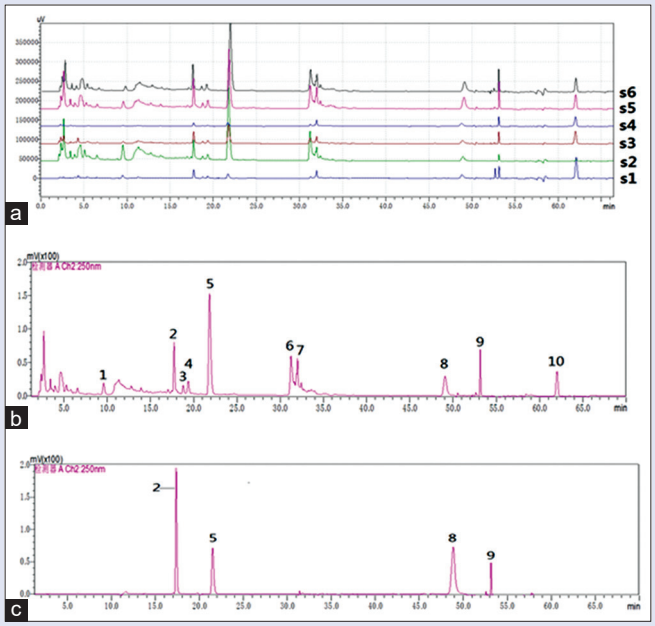

Figure 1: (a) High-performance liquid chromatography fingerprint; (b) the mutual mode (Methanol extract); and (c) mixed reference substances (S1–S6: ethyl acetate, Water, petroleum ether, acetone, ethanol, methanol. 2: β-ecdysterone, 5: Tetrahydroxystilbene glucoside, 8: Emodin, 9: Emodin methyl ether)

RESULTS

Result of the high-performance liquid chromatography experiment

Figure 1 shows the HPLC fingerprints of CPMAB and the chromatogram of reference substance. There were ten common peaks found by comparing the ultraviolet spectra and HPLC retention time from the samples. Four common peaks were identified by comparing the reference substances. They were as follows: $X2$: β-ecdysterone (t_R = 17.577), $X5$: Tetrahydroxystilbene glucoside (t_R = 21.930), $X8$: Emodin (t_R = 49.198), and $X9$: Emodin methyl ether (t_R = 53.167).

The relative retention time [Table 2], relative peak area [Table 3], and percentage coefficient of variance of the peak area of 15 common characteristic peaks are shown (tetrahydroxystilbene glucoside was selected as the reference). The relative SD percentage (RSD%) of relative retention time was < 0.9%, whereas RSD% of relative peak area was in the range of 12.48%–140.34%. The results showed that different extracting solvents had great influence on the dissolution of active ingredients of CPMAB. The results of SPSS Chi-square test showed that the relative peak areas of S3 (petroleum ether extract) and S1 (ethyl acetate extract) were significantly different when compared with other groups.

The reference solution was precisely absorbed and injected into the HPLC under the above chromatographic conditions [Table 1]. The standard curve was plotted [Table 4] (A is the injection volume and C is the area of chromatographic peak). According to our results, the linear relationship was good. The corresponding fingerprint chromatographic peak areas of different extracting solvents were substituted into regression equation to calculate the actual content of each component. Table 5 shows the average results. The extraction efficiency of S2, S5, and S6 was higher from the content of the main components of the extract.

Table 2: The relative retention time of 10 common peaks

Sample	X1	X2	X3	X4	X5	X5	X7	X8	X9	X10
S1	0.438	0.817	0.865	0.893	1	1.441	1.473	2.249	2.447	2.859
S2	0.438	0.814	0.862	0.889	1	1.434	1.469	2.248	2.439	2.851
S3	0.439	0.814	0.862	0.889	1	1.432	1.468	2.245	2.438	2.845
S4	0.439	0.817	0.865	0.892	1	1.441	1.474	2.249	2.448	2.858
S5	0.439	0.812	0.859	0.887	1	1.431	1.466	2.247	2.434	2.841
S6	0.444	0.812	0.848	0.874	1	1.425	1.46	2.249	2.424	2.834
X	0.439	0.814	0.860	0.887	1	1.434	1.468	2.248	2.438	2.848
RSD%	0.514	0.276	0.741	0.776	1	0.432	0.346	0.071	0.364	0.346

Table 3: The relative peak area of 10 common peaks

Sample	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10
S1	0.568	1.03	0.231	0.365	1	0.25	0.936	1.532	1.936	4.54
S2	0.176	0.202	0.053	0.102	1	0.311	0.138	0.068	0.025	0.034
S3	0.121	0.432	0.091	0.16	1	0.26	0.279	0.471	0.285	0.365
S4	0.186	0.557	0.133	0.221	1	0.334	1.013	1.677	2.382	2.499
S5	0.112	0.325	0.071	0.117	1	0.335	0.265	0.281	0.172	0.208
S6	0.076	0.253	0.062	0.086	1	0.282	0.152	0.241	0.155	0.196
X	0.206	0.466	0.107	0.175	1	0.295	0.464	0.712	0.826	1.307
RSD%	88.05	65.19	62.85	59.91	0	12.48	86.33	99.04	126.60	140.34

RSD%: Relative standard deviation percentage

Table 4: Regression equation and linear range

Chemical composition	Regression equation	Correlation coefficient	Linear range (μg)
β-Ecdysterone	*C*=1,000,000A+129,247	0.9995	0.08-2.90
Tetrahydroxystilbene glucoside	*C*=325,656A+187, k436	0.9997	1.18-5.90
Emodin	*C*=2,000,000A+178,228	0.9996	2.36-16.8
Emodin methyl ether	*C*=181,550A+129,523	0.9994	0.760-4.10

Table 5: Content of four components in each extract (mg/g, *n*=5)

Sample	β-Ecdysterone	Tetrahydroxystilbene glucoside	Emodin	Emodin methyl ether
S1	1.699±0.241	6.575±0.478	0.141±0.017	22.973±1.853
S2	7.232±0.922	128.572±15.474	0.102±0.009	4.628±0.354
S3	2.759±0.225	25.202±1.879	0.152±0.013	11.449±1.408
S4	0.373±0.044	5.034±0.472	0.119±0.012	22.463±1.764
S5	8.972±0.887	97.319±8.156	0.370±0.022	26.734±2.781
S6	7.751±0.747	109.249±9.232	0.355±0.018	26.989±3.013

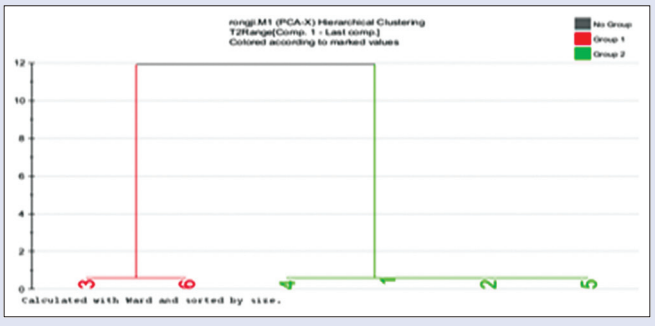

Figure 2: Results of hierarchical cluster analysis

Results of hierarchical cluster analysis

From the results of tree graph [Figure 2], S3 (acetone extract) and S6 (ethyl acetate extract) could be grouped into one group, and S1 (methanol extract), S2 (ethanol extract), S4 (petroleum ether extract), and S5 (water extract) could be grouped into another group. The results showed that the extraction effect of acetone and ethyl acetate was significantly different from that of the other solvents.

Result of the antiosteoporosis experiment

The average contents of BALP, TRACP-5b, and E2 in mice sera were detected by ELISA to evaluate the antiosteoporotic activity of CPMAB [Figure 3]. The results of BALP experiment showed that there was an increasing trend in the model group, and there was no significant difference between the model group and sham operation group.

The histomorphometry changes of tibias were investigated by H and E staining. As shown in Figure 4, the sham group presented with well-formed and competent trabecular bone. The results of the treatment group showed that the drug had a good anti-OP effect.

Results of correlation analysis

Table 6 shows the results of correlation analysis between fingerprint common peaks of different solvent extracts and anti-OP effect. It shows that CPMAB had the greatest effect on TRACP-5b, less on BALP, and the least on E2.

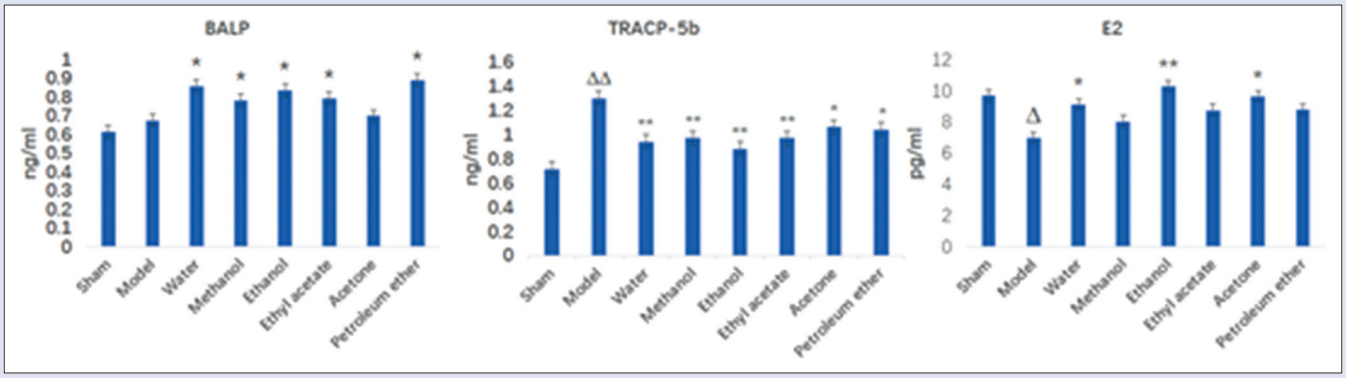

Figure 3: Determination of plasma factor by enzyme-linked immunosorbent assay ($n = 10$, Δ: $P < 0.05$ was significant difference between model group and the Sham group. ΔΔ: $P < 0.01$ was significant difference between model group and the Sham group. *$P < 0.05$ was significant difference between treatment groups and the model group. **$P < 0.01$ was significant difference between treatment groups and the model group)

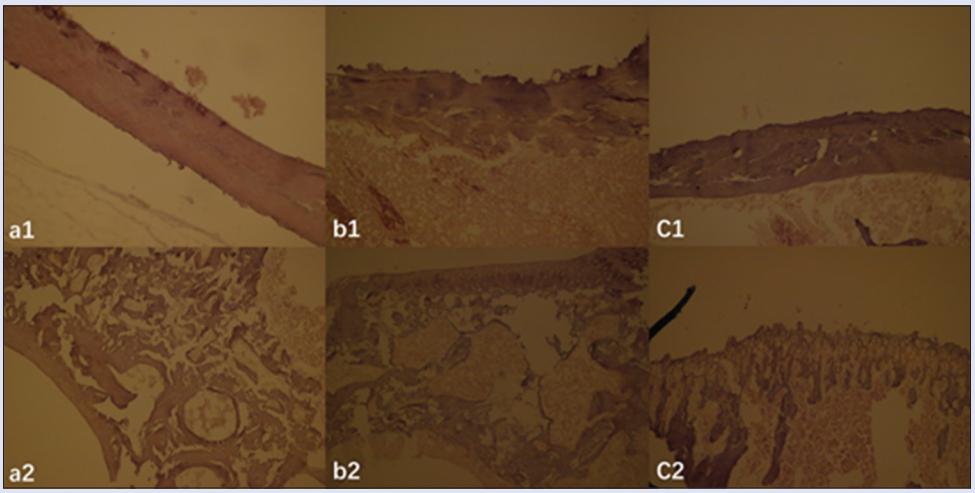

Figure 4: Effect of compatibility of *Polygoni multiflori-Achyranthes bidentate* on bone (left tibias, 200×) histomorphometry (a. Sham operation group, b. osteoporosis model group, c. osteoporosis treatment group, 1. Compact bone, 2. Cancellous bone)

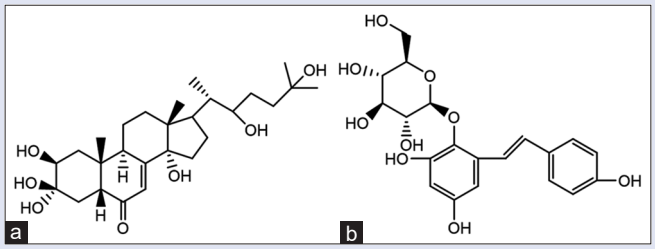

Figure 5: The structural formulas of β-ecdysterone (a) and tetrahydroxystilbene glucoside (b)

Table 7 shows the results of correlation analysis between the content of four components of different solvent extracts and anti-OP effect. It shows that the four components had the greatest effect on TRACP-5b, less on BALP, and the least on E2.

DISCUSSION

Compared with the sham-operated group, the cortical bone and cancellous bone in the model group were significantly reduced, the trabeculae in cancellous bone were reduced, and the marrow cavity

Table 6: Relevance analysis of common fingerprint peaks

fingerprint peaks	BALP	TRACP-5b	E2
X1	0.46	−0.645	0.087
X2	0.433	−0.826*	0.088
X3	0.411	−0.794	0.021
X4	0.494	−0.791	0.114
X5	0.38	−0.887*	−0.028
X6	0.385	−0.739	0.073
X7	0.332	−0.695	0.376
X8	0.04	−0.576	0.055
X9	−0.573	−0.17	0.083
X10	−0.371	−0.207	−0.153

*P<0.05 was significant correlation. BALP: Bone-specific alkaline phosphatase; TRACP-5b: Tartrate-resistant acid phosphatase 5b; E2: Estradiol 2

Table 7: Relevance analysis of four components

Chemical composition	BALP	TRACP-5b	E2
β-Ecdysterone	0.433	−0.806*	0.088
Tetrahydroxystilbene glucoside	0.38	−0.885*	−0.028
Emodin	0.041	−0.578	0.055
Emodin methyl ether	-0.573	−0.17	0.083

*P<0.05 was significant correlation. BALP: Bone-specific alkaline phosphatase; TRACP-5b: Tartrate-resistant acid phosphatase 5b; E2: Estradiol 2

was significantly enlarged, which indicated that the model of OP was successfully replicated. The bone mass of cortical bone and cancellous bone increased significantly in the treatment group, indicating that CPMAB had a significant therapeutic effect on OP induced by retinoic acid.

The results showed that the OP model replicated by retinoic acid had little effect on bone formation index, which is BALP. The levels of BALP were significantly increased in each treatment group. The order of activity of extracts from each group was as follows: petroleum ether > water > ethanol > ethyl acetate > methanol > acetone. The results of TRACP-5b experiment showed that, compared with the sham-operated group, the model group showed increased levels significantly. The levels of TRACP-5b in each treatment group were significantly decreased. The order of activity of extracts from each group was as follows: ethanol > water > methanol > ethyl acetate > petroleum ether ≈ acetone. The results of E2 experiment showed that compared with the sham-operated group, the model group showed increased levels significantly. The levels of E2 in each treatment group were significantly increased. The order of activity of extracts from each group was as follows: ethanol > acetone > water > petroleum ether ≈ ethyl acetate > methanol.

Results of correlation analysis showed that the order of influence on TRACP-5b was as follows: *X*5>*X*2>*X*3 > *X*4>*X*6>*X*1 > *X*7>*X*8>*X*10>*X*9. Among them, *X*2 and *X*5 showed significant activity. The order of effect on BALP was as follows: *X*4>*X*1>*X*2 > *X*3>*X*6>*X*5 > *X*7>*X*8>*X*10>*X*9. Among them, *X*9 and *X*10 were negatively correlated. The area of each chromatographic peak was negatively correlated with TRACP-5b and the peak *X*2 (β-ecdysterone) and *X*5 (tetrahydroxystilbene glucoside) were highly negatively correlated. The higher the content of *X*2 (β-ecdysterone) and *X*5 (tetrahydroxystilbene glucoside) in the extract, the lower the content of TRACP-5b. It may also indicate that the anti-OP mechanism of CPMAB may be through decreasing the level of TRACP-5b.

The results of correlation analysis between chemical composition and pharmacodynamics were consistent with those of common peak area of fingerprints. β-Ecdysterone and tetrahydroxystilbene glucoside may contribute a lot to the anti-OP effect of extracts.

Our results showed that the HPLC fingerprints of CPMAB extracted by different solvents were significantly different, and the anti-OP effect was significantly different. In this study, the spectrum–effect relationship between the relative common peak area of HPLC fingerprint and the activity of anti-OP, the content of main components, and the activity of anti-OP were established. The results of correlation analysis showed that β-ecdysterone and tetrahydroxystilbene glucoside [Figure 5] were highly correlated to TRACP-5b.

CONCLUSION

According to our results, CPMAB exhibits anti-OP effect. It showed this activity by reducing the number of osteoclasts. β-Ecdysterone and tetrahydroxystilbene may be the active components of CPMAB in anti-OP. This study provides a reference for further study on the compatibility mechanism and active components of CPMAB.

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Conflicts of interest

There are no conflicts of interest.

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