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Inhibitory effect of constituents of Bu-Shen-Jian-Gu-Tang on osteoclast-like cell formation

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The constituents of Bu-Shen-Jian-Gu-Tang and their inhibitory activities on osteoclast-like cell formation were investigated. Bu-Shen-Jian-Gu-Tang was fractionated by a Diaion HP-20 column with EtOH-H_2O, and the 60% and 90% EtOH-H_2O fractions showed more potent inhibitory activities on osteoclast-like cell formation and bone resorption. Chemical investigation of these two fractions resulted in the isolation of 11 compounds, for which the structures were elucidated based on spectroscopic analysis and chemical reactions. The inhibitory activities of the isolated compounds on osteoclast-like cell formation were evaluated. Among the eleven compounds, icariin (1), icarisid II (2), 2"-*O*-rhamnosylicariside II (3), kaempferol (8), chikusetsusaponin IVa (10) and chikusetsusaponin V (11) showed potent inhibitory activities at a concentration of 2 μM without toxic effects. The preventive effect of Bu-Shen-Jian-Gu-Tang on bone loss may be due to the inhibitory activities of these compounds.

Key words antiosteoporotic activity, Kampo formula, Bu-Shen-Jian-Gu-Tang, osteoclast, inhibitory activity.

Introduction

Osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture.[1] In traditional Chinese medicines, formulations using herbal interaction are often used to treat bone disease. It is reasonable, therefore, to postulate that these formulations may affect the process of bone metabolism. Thus, we investigated 19 Chinese herbal formulations used to treat bone diseases in clinics in China for their inhibitory activities on osteoclast-like cell formation and bone resorption *in vitro*.[2] Among the 19 Kampo formulae, Bu-Shen-Jian-Gu-Tang showed the most potent inhibitory activities on osteoclast-like cell formation and bone resorption. Further *in vivo* experiments in ovariectomy-induced postmenopausal osteoporosis model rats revealed a preventive effect on bone loss.[2] However, there is no report on its bioactive constituents. Thus, we examined the constituents of Bu-Shen-Jian-Gu-Tang and their inhibitory effect on osteoclast-like cell formation.

Materials and Methods

General. Mice (ddY) were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, Kansas, USA), and alpha modified Eagle's medium (α-MEM) and horse serum were obtained from ICN Biomedicals, Inc. (Aurora, Ohio, USA). Ham's F-12 medium was purchased from Nissui Seiyaku Co. (Tokyo, Japan). Parathyroid hormone (human, 1-34, PTH) was obtained from Peptide Institute, Inc. (Osaka, Japan), elcatonin from Asahi Kasei Corporation (Osaka, Japan) and ^{45}Ca (772.34 MBq/mg) from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA, USA). All other reagents were from Wako Pure Chemicals Industries, Ltd. (Kyoto, Japan).

NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard. Diaion HP-20 was purchased from Mitsubishi Chemical Corporation (Tokyo, Japan). Column chromatography was performed with Cosmosil 75C_{18}-OPN (Nacalai Tesque, Inc., Kyoto, Japan). Medium pressure liquid chromatography (MPLC) was performed with an ODS-P-40C column (3.7 × 30 cm) (Yamazen Corporation, Osaka, Japan). HPLC was performed with a Supelco Discovery® C_{18} column (250 × 10 mm i.d., 5 μm) (Bellefonte, PA, USA). Analytical and preparative TLC was carried out on precoated silica gel 60F_{254} and RP-18F_{254} plates (Merck, 0.25 mm thickness).

Extraction and isolation. The prescription of Bu-Shen-Jian-Gu-Tang is composed of Radix Rehmanniae Preparata 20 g, Fructus Corni 10 g, Semen Cuscutae 10 g, Radix Achyranthis Bidentatae 10 g, Herba Epimedii 10 g, Herba Cistanches 10 g, Rhizoma Dioscoreae 15 g, Radix Salviae Miltiorrhizae 15 g, Fructus Lycii 8 g, Radix Notoginseng 3 g, Colla Cornus Cervi 10 g and Colla Carapacis et Plastri Testudinis 10 g.[3] Each component of this formula was purchased from Chengdafangyuan Drug Store, Dalian, China, in January, 2005.

Bu-Shen-Jian-Gu-Tang was prepared as described by Ye.[4] A mixture (1110 g) of the components in the same ratio as above, except for Colla Cornus Cervi and Colla

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Carapacis et Plastri Testudinis, was extracted with a three-fold volume of water (reflux, 1 h, ×3), then Colla Cornus Cervi (100 g) and Colla Carapacis et Plastri Testudinis (100 g) were added to the extract. The mixture was concentrated under reduced pressure and lyophilized to give Bu-Shen-Jian-Gu-Tang (510 g). Bu-Shen-Jian-Gu-Tang (400 g) was fractioned by a Diaion HP-20 column (8 × 60 cm) with H_2O and 30%, 60% and 90% EtOH-H_2O to give four fractions (H_2O fraction, 235 g; 30% EtOH-H_2O fraction, 74.4 g; 60% EtOH-H_2O fraction, 16.1 g; 90% EtOH-H_2O fraction, 2.4 g). The 60% and 90% EtOH fractions, which showed potent inhibitory activities both on osteoclast-like cell formation and bone resorption, were subjected to further isolation.

The 60% EtOH fraction (9.1 g) was subjected to column chromatography (5 × 25 cm) on Cosmosil 75C_{18}-OPN with acetone-CH_3CN-H_2O (1:1:3) to give three fractions (fraction 1, 2.4 g; fraction 2, 1.1 g; fraction 3, 110 mg). Fraction 1 was subjected to MPLC with acetone-CH_3CN-H_2O (1:1:3) to give three subfractions (fraction 1-1, 50 mg; fraction 1-2, 146 mg, fraction 1-3, 171 mg). Each fraction was separated by preparative TLC with BuOH-EtOAc-H_2O (4:1:2, upper layer), and **9** (10.8 mg), **1** (54.2 mg) and **6** (7.8 mg) were obtained from fractions 1-1, 1-2 and 1-3, respectively. Fraction 2 was subjected to MPLC with acetone-CH_3CN-H_2O (1:1:2) to give two subfractions (fraction 2-1, 95 mg; fraction 2-2, 90 mg). Each fraction was separated by reversed-phase preparative TLC with acetone-CH_3CN-H_2O (1:1:2), and **7** (2.1 mg) and **8** (4.5 mg) were obtained from fractions 2-1 and 2-2, respectively. Fraction 3 was separated by preparative TLC with BuOH-EtOAc-H_2O (4:1:2, upper layer) to give **3** (5.2 mg), **4** (2.3 mg) and **10** (2.3 mg).

The 90% EtOH fraction (1.8 g) was subjected to column chromatography (3.5 × 18 cm) on Cosmosil 75C_{18}-OPN with acetone-CH_3CN-H_2O (1:1:1) to give four fractions (fraction 1, 211 mg; fraction 2, 575 mg; fraction 3, 175 mg; fraction 4, 598 mg). Fraction 1 was subjected to MPLC with acetone-CH_3CN-H_2O (1:1:3) to give two subfractions (fraction 1-1, 63 mg; fraction 1-2, 26 mg). Each fraction was separated by reversed-phase preparative TLC with acetone-CH_3CN-H_2O (1:1:3), and **6** (1.2 mg) and **11** (4.5 mg) were obtained from fraction 1-1 and **10** (1.5 mg) was obtained from fraction1-2. Fraction 2 was subjected to MPLC with acetone-CH_3CN-H_2O (1:1:3) to give two subfractions (fraction 2-1, 18 mg; fraction 2-2, 82 mg). Fraction 2-1 was separated by HPLC with MeOH-CH_3CN-H_2O (1:1:4) to give **5** (1.0 mg) and **6** (2.3 mg). Fraction 2-2 was separated by preparative TLC with $CHCl_3$-MeOH-H_2O (12:5:1) to give **1** (11.2 mg). Fraction 3 was separated by preparative TLC with $CHCl_3$-MeOH-H_2O (12:5:1) to give **1** (5.6 mg). Fraction 4 was subjected to MPLC with acetone-CH_3CN-H_2O (1:1:3) to give three subfractions (fraction 4-1, 187 mg; fraction 4-2, 65 mg, fraction 4-3, 108 mg). Fraction 4-1 was separated by reversed-phase preparative TLC with acetone-CH_3CN-H_2O (1:1:3) to give **1** (18.6 mg) and **11** (3.4 mg). Fraction 4-2 was separated by preparative TLC with BuOH-EtOAc-H_2O (4:1:2, upper layer) to give **10** (11.4 mg). Fraction 4-3 was subjected to HPLC with MeOH-CH_3CN-H_2O (1:1:4) to give **2** (5.6 mg), **3** (3.3 mg) and **4** (1.5 mg).

Hydrolysis of icariin (1). A solution of icariin (**1**, 15 mg) in 1 N HCl (dioxane-H_2O, 1:1; 2 ml) was heated at 80 ℃ for 4 h. Water was added to the reaction mixture, and the entire mixture was extracted with EtOAc and concentrated. The residue was separated by preparative TLC with CH_3Cl-MeOH (95:5) to yield icaritin (**12**, 3.6 mg).

Bone marrow cell culture. Inhibitory activity on osteoclast-like cell formation was assessed using the method of Takahashi *et al.*[5] Osteoclast-like multinucleated cells were originated from a culture of mouse bone marrow cells. The marrow cavities of the tibiae of male ddY mice (seven weeks old) were flushed with α-MEM, and the bone marrow cells were cultured in α-MEM containing 10% FBS at 1.5×10^6 cells/ml in 24-well plates (0.5 ml/well) in the presence of PTH (24 nM) and test samples at various concentrations. Elcatonin (2 U/ml) was used as a positive control. The medium was replaced with new medium containing test samples and PTH every three days. All cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. After culture for eight days, cells adherent to the well surface were fixed with 10% formalin in phosphate-buffered saline (PBS, pH 7.4) for 10 min and then treated with ethanol-acetone (1:1) for one min. The cells were stained with tartrate-resistant acid phosphatase (TRAP) staining solution. TRAP-positive multinucleated cells containing three or more nuclei were recognized as osteoclast-like cells and counted.

Reversibility assay. A reversibility assay was performed as described above. The cells were treated with PTH (24 nM) and each compound for the first four days, and thereafter each compound was removed from the medium and the cells were cultured with PTH (24 nM) only.[6,7]

Assay of bone-resorbing activity *in vitro*. Bone-resorbing activity was assessed using the method of Shigeno *et al.*[8] ^{45}Ca (0.074 MBq) was injected subcutaneously into ddY mice (two days old). Two days later, half parietal bones were taken out and cultured in sterile plastic 24-well culture plates on stainless steel grids on the top of glass rings that supported the bones near the gas-liquid interface. Bones were cultured in Ham's F-12 medium (1 ml/well) supplemented with 0.2% $NaHCO_3$, 1.0 mM $CaCl_2$, 25 μg/ml streptomycin, 15 μg/ml penicillin, and 5% heat-inactivated horse serum in an incubator at 37℃ under 5% CO_2 in air. Bones were randomly assigned to a control or treatment group and each group included four bones. Bones were incubated for a total of 168 h; the first 24 h allowed for efflux into the medium of readily exchangeable ^{45}Ca in bone. After being cultured for 24 h and 96 h, bones were transferred to the new medium in the presence of PTH (2 nM) and test samples at various concentrations. Elcatonin (4 U/ml) was used as a positive control. After being cultured for 168 h, bones were extracted for three days with 0.01 M EDTA-acetate buffer solution (pH 5.5). The radioactivity of ^{45}Ca released into the culture medium from parietal bones at 96 h and 168 h and into the EDTA solution was measured by a Beckman LS 3801 liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA). Bone resorption was quantified on the

$$^{45}\text{Ca released (\%)} = \frac{\text{Total } ^{45}\text{Ca released into the medium}}{\text{Total } ^{45}\text{Ca released into the medium} + ^{45}\text{Ca released into the EDTA solution}} \times 100$$

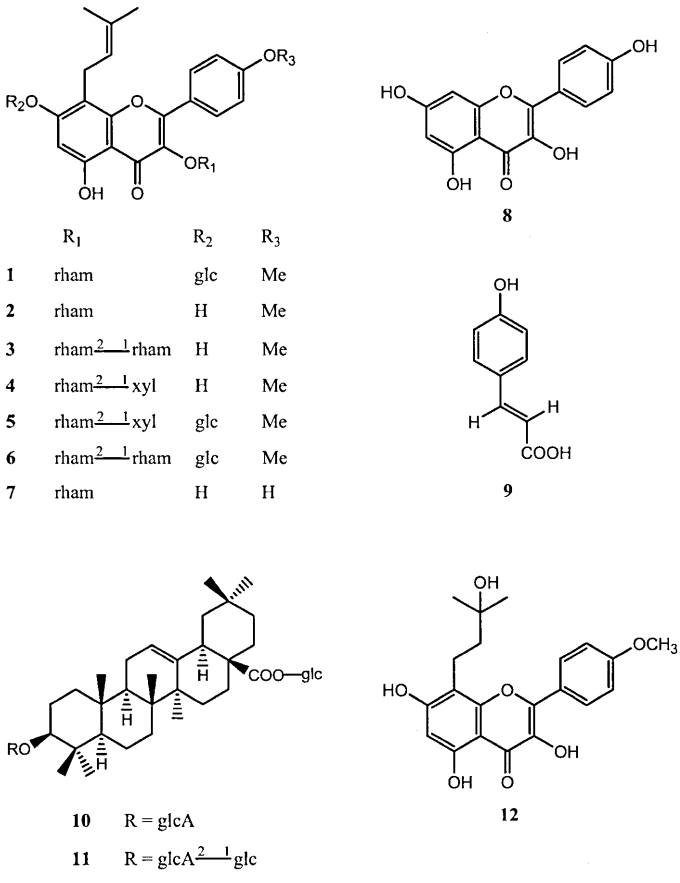
basis of the percentage of bone ^{45}Ca released into the medium.

Ethical considerations. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Toyama.

Statistical analysis. The data were analyzed by one way analysis of variance (ANOVA) for multiple comparisons between groups. Values are expressed as the mean ± SD. Differences with $p < 0.05$ were considered statistically significant.

Results and Discussion

In order to examine the active constituents of Bu-Shen-Jian-Gu-Tang, we examined its effect on PTH-induced formation of osteoclast-like cells and PTH-induced bone resportion. When mouse bone marrow cells were cultured in the presence of PTH, tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells were formed; these were recognized as osteoclast-like cells, the primary cells responsible for bone resorption.[9,10] The osteoclast-like cell formation (%) of each group was calculated relative to the PTH group. The inhibitory activity on bone resorption was indicated as ^{45}Ca release (%) from neonatal mouse parietal bone. As shown in Table 1, Bu-Shen-Jian-Gu-Tang showed potent inhibitory activities on osteoclast-like cell formation (2.3% formation) and bone resorption (44.5% of ^{45}Ca release) at a concentration of 500 μg/ml. Accordingly, Bu-Shen-Jian-Gu-Tang was fractionated by column chromatography over Diaion HP-20 with H_2O and 30%, 60% and 90% EtOH-H_2O to give four fractions. The 60% EtOH and 90% EtOH fractions showed potent inhibitory activities on osteoclast-like cell formation (3.9% and 3.1% formation, respectively) and bone resorption (45.5% and 30.2% of ^{45}Ca release, respectively) at a concentration of 500 μg/ml (Table 1). Therefore, chemical investigation of these two fractions was carried out and led to isolation of 11 compounds. Their structures were elucidated based on the spectroscopic analysis and comparison with published data as icariin (**1**),[11] icarisid II (**2**),[12] 2"-*O*-rhamnosyl icariside II (**3**),[13] sagittatoside B (**4**),[14] epimedin B (**5**),[15] epimedin C (**6**),[15] ikarisoside A (**7**),[16] kaempferol (**8**),[17] 4-hydroxycinnamic acid (**9**),[18] chikusetsusaponin IVa (**10**)[19] and chikusetsusaponin V (**11**)[19] (Fig. 1). These 11 compounds were tested for their inhibitory activity on osteoclast-like cell formation (Table 2). Among them, icariin (**1**), icarisid II (**2**), 2"-*O*-rhamnosylicariside II (**3**), kaempferol (**8**), chikusetsusaponin



	R_1	R_2	R_3
1	rham	glc	Me
2	rham	H	Me
3	rham(2→1)rham	H	Me
4	rham(2→1)xyl	H	Me
5	rham(2→1)xyl	glc	Me
6	rham(2→1)rham	glc	Me
7	rham	H	H

8 **9**

10 R = glcA

11 R = glcA(2→1)glc

12

Fig. 1

Table 1. Inhibitory activities of the fractions on osteoclast-like cell formation and bone resorption

Sample	Osteoclast-like cell formation (%)		^{45}Ca release (%)	
	50 μg/ml	500 μg/ml	50 μg/ml	500 μg/ml
Bu-Shen-Jian-Gu-Tang	19.0 ± 5.9**	2.3 ± 3.0**	77.5 ± 7.8	44.5 ± 9.4**
0% EtOH fraction	37.2 ± 14.5**	4.6 ± 5.9**	82.9 ± 9.8	84.1 ± 7.2
30% EtOH fraction	45.7 ± 11.1**	0.0 ± 0.0**	91.3 ± 7.0	85.5 ± 12.0
60% EtOH fraction	13.9 ± 8.2**	3.9 ± 7.7**	81.3 ± 12.0	45.5 ± 4.3**
90% EtOH fraction	18.6 ± 9.1**	3.1 ± 6.2**	39.5 ± 3.6**	30.2 ± 2.0**
Elcatonin	28.2 ± 11.8** (2 U/ml)		56.3 ± 8.8* (4 U/ml)	
PTH	100.0 ± 25.4 (24 nM)		76.7 ± 6.5 (2 nM)	

Osteoclast-like cell formation: bone marrow cells were cultured with PTH (24 nM). Bone resorption: bone was cultured with PTH (2 nM). Each value is expressed as the mean ± SD, n = 4. Significant differences compared with the PTH group, *$p < 0.05$, **$p < 0.01$.

Table 2. Inhibitory activities of the isolated compounds on osteoclast-like cell formation

Compound	Osteoclast-like cell formation (%)		
	2 μM	20 μM	200 μM
1	19.5 ± 14.3**	11.0 ± 7.3**	4.9 ± 9.8**
2	8.6 ± 2.4**	4.9 ± 5.6**	0.0 ± 0.0**
3	23.2 ± 18.9**	18.3 ± 17.5**	0.0 ± 0.0**
4	29.2 ± 8.9**	21.9 ± 6.3**	0.0 ± 0.0**
5	70.7 ± 56.4	30.5 ± 12.2**	0.0 ± 0.0**
6	36.6 ± 28.0*	22.0 ± 19.7**	7.3 ± 9.3**
7	63.4 ± 40.3	20.7 ± 16.1**	0.0 ± 0.0**
8	12.2 ± 9.3**	7.3 ± 2.8**	0.0 ± 0.0**
9	57.3 ± 44.6	34.1 ± 25.5*	28.1 ± 27.7*
10	15.9 ± 12.2**	14.7 ± 9.7**	1.2 ± 2.5**
11	24.8 ± 16.1*	14.4 ± 11.7*	12.4 ± 9.3**
icaritin	48.8 ± 38.6	23.2 ± 23.7*	0.0 ± 0.0**
Elcatonin	24.8 ± 14.1** (2 U/ml)		
PTH	100.0 ± 22.1 (24 nM)		

PTH: cultured with PTH (24 nM); Elcatonin: cultured with PTH (24 nM) and elcationin (2 U/ml); Samples: cultured with PTH (24 nM) and each compound. The data were calculated relative to the PTH group. Each value is expressed as the mean ± SD, n = 4. Significant differences in osteoclast-like cell formation compared with the PTH group, $*p < 0.05$, $**p < 0.01$.

Table 3. Reversibility of inhibitory effects of the compounds on osteoclast-like cell formation

Compound	Osteoclast-like cell formation (%)		Osteoclast-like cell formation (%)	
	0-8 d		0-4 d	
	2 μM	20 μM	2 μM	20 μM
1	24.8 ± 14.2**	18.6 ± 5.4**	84.6 ± 41.2	72.2 ± 27.0
2	13.4 ± 6.4**	0.0 ± 0.0**	77.4 ± 6.2	54.7 ± 10.9
3	26.8 ± 17.1**	21.7 ± 10.7**	63.0 ± 31.6	29.9 ± 14.0
8	13.4 ± 1.8 **	5.2 ± 8.9**	51.6 ± 10.0	50.6 ± 15.6
10	23.7 ± 14.6**	17.5 ± 6.4**	99.1 ± 56.8	52.6 ± 49.1
11	24.8 ± 16.1**	14.4 ± 11.7**	64.0 ± 23.2	38.2 ± 22.8

0-8 d: bone marrow cells were cultured with PTH (24 nM) and each compound for eight days. 0-4 d: the cells were treated with PTH (24 nM) for eight days and each compound for four days. The data were calculated relative to the PTH group. Each value is expressed as the mean ± SD, n = 4. Significant differences compared with the PTH group, $*p < 0.05$, $**p < 0.01$.

IVa (**10**) and chikusetsusaponin V (**11**) showed potent inhibitory activity on osteoclast-like cell formation at a concentration of 2 μM. As for the structure-activity relationship, the glucopyranosyl substituent on the hydroxyl group at C-7 decreased the inhibitory activity (**1** < **2**, **6** < **3**, **5** < **4**), while the methoxyl group at C-4' seemed to increase the inhibitory activity (**2** > **7**). As for the substituent at C-2 of the 3-*O*-rhamnopyranosyl group, glucose seemed to be more potent than xylose (**3** > **4**, **6** > **5**).

We should remember that the inhibitory activity of isolated compounds is sometimes a result of toxic effects. Thus, to clarify the toxicity of the active compounds, the reversibility of the inhibitory effects of the compounds **1-3**, **8**, **10** and **11**, which showed more potent inhibitory activity on osteoclast-like cell formation, was examined. As shown in Table 3, by removal of the test compound on the fourth day, the suppressive effects on osteoclast-like cell formation were recovered at a concentration of 2 μM, indicating the inhibitory activities at 2 μM were not due to their toxic effects. On the other hand, at 20 μM the suppressive effect of **3** and **11** were not recovered and other compounds showed only partial recovery. Thus, at a high concentration, they revealed little toxicity.

Compounds **1-3**, **8**, **10** and **11** showed significant inhibitory activity on osteoclast-like cell formation, and they did not possess irreversible toxic effects at a concentration of 2 μM. Thus, the preventive effect of Bu-Shen-Jian-Gu-Tang on bone loss seems to be due to the inhibitory activity of these compounds. Although more detailed experiments are needed, from the facts that icaritin (**12**), a hydrolysis product of icariin (**1**), showed inhibitory activity weaker than **1**, and that icariin (**1**) was detected in the serum after oral administration of **1**,[20] icariin itself may contribute to the antiosteoporotic activity *in vivo*.

Compounds **1-7** are reportedly contained in Herba Epimedii,[11-16] and the antiosteoporotic activity of Herba Epimedii was also reported.[21] Moreover, icariin (**1**) was reported to significantly promote osteoblast-like cell proliferation.[22] Chikusetsusaponin IVa (**10**) is contained in Radix Achyranthis Bidentatae[7] and is reported to inhibit osteoclast-like cell formation.[7] In this study, compounds **1-3**, **10** and **11**, which are reportedly contained in Herba Epimedii and Radix Achyranthis Bidentatae, showed potent inhibitory activities on osteoclast-like cell formation. Thus, Herba Epimedii and Radix Achyranthis Bidentatae may be the principal components of this formulation.

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Japanese abstract

本研究では，補腎健骨湯について，Diaion HP-20カラムクロマトグラフィを用いて，H_2O，30%，60%及び90% EtOH-H_2Oに順次分画し，各フラクションの破骨細胞形成及び骨吸収の抑制活性を検討したところ，60%及び90% EtOH-H_2O溶出部に抑制活性が強いことが判った。

さらに，これらのフラクションから単離した11種の化合物について，破骨細胞形成抑制活性を検討した。11種の化合物のうち，6種の化合物（icariin (**1**)，icarisid II (**2**)，2"-*O*-rhamnosylicariside II (**3**)，kaempferol (**8**)，chikusetsusaponin IVa (**10**) および chikusetsusaponin V (**11**)）が補腎健骨湯の骨吸収抑制活性成分であることが明らかとなった。

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