# The effect of traditional medicines on bone resorption induced by parathyroid hormone (PTH) in tissue culture: A detailed study on Cimicifugae Rhizoma[1)]

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## Abstract

Thirty-four MeOH and water extracts of natural crude drugs were screened for their inhibitory activities on bone resorption induced by parathyroid hormone (PTH) in bone organ culture. Thirteen MeOH extracts and nine water extracts showed significant inhibitory activities. Among these, the MeOH extract of *Cimicifuga heracleifolia* KOMAROV and *C. foetida* L. showed a potent inhibitory activity, so that MeOH extracts of these two species were further fractionated into hexane, EtOAc, *n*-BuOH, and water soluble fractions. Each fraction and sixteen triterpenoids isolated from EtOAc and *n*-BuOH fractions were performed for the bone resorption assay. On the basis of a structure-activity relationship analysis of inhibitory activity on bone resorption, the triterpenoids were considered to contribute to the inhibitory activity of Cimicifugae Rhizoma on bone resorption.

**Key words** Cimicifugae Rhizoma, bone resorption, triterpenoids, plant crude drugs, bone organ culture, parathyroid hormone, ipriflavone.

**Abbreviations** PTH, parathyroid hormone; LH, luteinizing hormone ; ECF, extracellular fluid.

## Introduction

Osteoporosis is a state of low bone mass, which is the leading cause of bone fracture in the old person, especially, the menopausal woman.[2)] Patients with established osteoporosis visit physicians with various complaints including back pain and weakness of their back. Also for the aged, it produces bedridden patients, with resulting dementia and pneumonia. However, only a few drugs, including estrogen, 1 $\alpha$, 25-dihydroxyvitamin $D_3$, 1 $\alpha$-hydroxyvitamin $D_3$, ipriflavone (TC-80) and elcatonin (eel calcitonin) are available for the treatment of osteoporosis in clinic.[3)] The parathyroid hormone (PTH) plays important roles in the regulation of the calcium metabolism of bone. The bone organ culture system in which PTH was used as a bone resorption stimulator is widely used to evaluate the direct effect of drugs on bone.[4-6)] According to the theory of traditional Chinese medicine, those crude drugs with strengthening bones could be used for the treatment of bone diseases. Unfortunately no sufficient pharmacological studies are available. Therefore, an attempt was made to search for natural products possessing anti-osteoporosis activity. In the course of finding an anti-osteoporosis agent, we screened 34 extracts from 18 natural crude drugs which have been used in the traditional Chinese medicine as well as in Pakistan. The MeOH extracts of Cimicifugae Rhizoma was one of the potent inhibitors on bone resorption.

The Cimicifugae Rhizoma, "Shengma", (Ranunculaceae), which encompasses three *Cimicifuga* species namely *C. heracleifolia, C. dahurica,* and *C. foetida* is officially listed in the Chinese Pharmacopoeia.[7)] It is an important drug in traditional Chinese

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medicines for the treatment of inflammation, pain, and gynecological diseases, for example, descensus and prolapsus of uterus.[8] It was also reported that the ethanolic extract from *C. racemosa* (L.) NUTT. inhibited gonadotropin release activity in menopausal women and reduces luteinizing hormone (LH) secretion in ovariectomized rats.[9] But there is no report on its anti-osteoporosis activities.

The chemical constituents of *Cimicifuga* species have been studied by several groups.[10-12] In the previous papers,[13-15] we have reported the isolation and structure elucidation of some compounds from rhizomes of *C. heracleifolia* KOMAROV and *C. foetida* L.. In this paper, we wish to report the inhibitory activity of extracts and their fractions of *C. heracleifolia* and *C. foetida* on bone resorption, together with the activity of 17 constiuents isolated from methanol extract of Cimicifugae Rhizoma.

## Materials and Methods

*Animal and reagents*: Mice (Std: ddy) were purchased from Shizuoka Animal Center (Shizuoka, Japan). Parathyroid hormone (human, 1-34; PTH) was purchased from Peptide Institute, Inc. (Osaka, Japan). Ham's F-12 medium was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). $^{45}CaCl_2$ was purchased from NEN Research Products (Boston, MA). Ipriflavone was extracted and purified from commercially available Osten (TC-80) (Takeda Pharmaceutical Co. Ltd., Osaka, Japan). Cosmosil $140C_{18}$-OPN was from Nacalai Tesque, Inc. (Kyoto, Japan). Iatrobeads was purchased from Iatron Laboratories, Inc. (Tokyo, Japan). All other reagents were from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

*Plant materials*: All of the natural crude drugs used in the experiments were purchased from Tochimoto Tenkaidou (Osaka, Japan), except for *C. heracleifolia* and *C. foetida*, which were collected in China. A voucher specimen of each plant is deposited at the Museum of Materia Medica of Toyama Medical and Pharmaceutial University, Toyama, Japan.

*Preparation of extracts*: In general, the powdered natural crude drugs (200 g, each) were extracted with methanol (300 ml×2) by refluxing for 3 h in the first and second extractions. The hot extracts were filtered through filter paper and the combined filtrates were concentrated under reduced pressure, and lyophilized. The water extract of natural crude drugs was obtained by extracting with distilled water as described above.

*Fractionation of MeOH extracts from Cimicifugae Rhizoma and isolation of compounds*: The MeOH extracts of *C. heracleifolia* KOMAROV, collected at Heilongjiang Province, and *C. foetida* L., at Shichun Province of China, were suspended in water (300 ml) and fractionated by successive extraction with hexane, EtOAc, and *n*-BuOH to give hexane-, EtOAc-, *n*-BuOH-, and water-soluble fractions, respectively.

Detailed isolation and chemical structure of the pure compounds from EtOAc-soluble fractions were described in our previous papers.[13-15]

*i) Isolation of n-BuOH-soluble fraction from C. heracleifolia*: A part of *n*-BuOH-soluble fraction (5.0 g) was passed through a charcoal column with MeOH to give eluate (4.0 g). MeOH eluate was chromatographed on Iatrobeads column with $CHCl_3$-MeOH (3 : 2, 1 : 1, and 1 : 4) to give fraction I (0.7 g), II (2.0 g), and III (1.0 g). Fraction I (0.7 g) was subjected to a silica gel column chromatography with hexane-EtOAc-MeOH (2 : 4 : 0.5) to give 2',4'-*O*-diacetyl-24-*epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**2**) (6 mg). Fraction II (1.0 g) was rechromatographed on Iatrobeads column with $CHCl_3$-MeOH (3 : 2 and 1 : 1) to give two fractions. The first eluate fraction (0.5 g) gave 24-*epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**4**) (8 mg). The second fraction (0.3 g) was further purified by reversed-phase TLC to give 7,8-didehydro-24-*O*-acetylhydroshengmanol-3-*O*-$\beta$-xyloside (**8**) (5 mg). Fraction III (0.5 g) separated by reversed-phase TLC developed with MeOH-$H_2O$ (2 : 1) to give 8 (5 mg).

*ii) Isolation of n-BuOH-soluble fraction from C. foetida*: A part of *n*-BuOH-soluble fraction (5.0 g) was chromatographed on Cosmosil 140 $C_{18}$ column with MeOH-$H_2O$ (2 : 3 and 3 : 2) to give the 4 fractions, I (0.5 g), II (1.0 g), III (1.4 g), and IV (1.8 g). Fraction I (0.4 g) was subjected to a silica gel column chromatography with $CHCl_3$-MeOH (6 : 1) to give 25-anhydrocimigenol-3-*O*-$\beta$-xyloside (**6**) (5 mg) and *prim*-*O*-glucosylagelicain (3 mg). Fraction II (0.5 g) was rechromatographed on Cosmosil 140 $C_{18}$ column with MeOH-$H_2O$ (2 : 3) to give acetylacteol-3-*O*-

arabinoside (**10**) (8 mg). Fraction III and IV (each 1.0 g) were rechromatographed by same method as fraction II, giving cimicidanol-3-*O*-$\beta$-xyloside (**12**) (10 mg) and cimicidol-3-*O*-$\beta$-xyloside (**13**) (15 mg), respectively.[16]

All isolated triterpenoids were identified by comparison with an authentic sample.[13,15] *prim*-*O*-Glucosylagelicain was confirmed by comparison with $^1$H- and $^{13}$C-NMR and specific rotation with data in the literature.[17]

In the assay, all of the tested samples except for water extracts were dissolved in DMSO and added at a DMSO cocentration of 0.1 %. DMSO at 0.1 % was added to PTH, normal and water extracts groups and did not affect bone resorption.

*Bone organ culture system* : The bone-resorbing activity assay reported by Shigeno *et al.* was used.[18] Briefly, 2-day-old mice were injected subcutaneously with $^{45}CaCl_2$ (2 $\mu$Ci). Two days later, the parietal bones were taken out under ether anesthesia and cultured in sterile plastic multiwell culture plates (well area, 2 cm$^2$) on stainless steel grids on top of glass rings that supported the bones near the gas-liquid interface. Ham's F-12 medium (1 ml / well), which consist of 10.6 g/liter Ham's F-12 medium, 2.2 g/liter $NaHCO_3$, 1.0 mM $CaCl_2$, and 5 % (v/v) heat-inactivated horse serum, were used. Bones were incubated at 37°C and in an incubator with 5 % $CO_2$.

Bones were randomly assigned to control and treated groups and each group consisted of six or seven bones. After pre-culturing for 24 h, the medium was removed and a fresh medium with PTH (final concentration, $2 \times 10^{-9}$M) and samples tested were added, then bones were incubated for 144 h. In the experimental period, after 72 h, the medium was changed with the fresh medium. After finishing the culture, bones were removed and put in a 0.01 M EDTA-acetic acid buffer solution (pH=5.5) to desert $^{45}$Ca contained in bone. $^{45}$Ca released into the culture medium from prelabeled bones at 72 h and 144 h and the EDTA solution containing in prelabeled bones were counted separately. Bone resorption was quantified on the basis of percentage of $^{45}$Ca released into the medium to the total $^{45}$Ca.

*Statistical Analysis* : Significance of the mean differences in the bone resorption assay was analyzed by Student's *t* test

## Results and Discussion

PTH plays a major role on calcium homeostasis. It restores normal calcium concentration in extracellular fluid (ECF) by acting directly on bone and increases the rate of dissolution of bone, including both organic and inorganic phases. Therefore, the addition of PTH causes calcium release from bones in continuous exposure *in vitro*. The experiment demonstrated that the release of calcium from PTH-treated bones was significantly increased as compared with the normal group.

We have examined the inhibition effect of 34 extracts from 18 natural crude drugs on bone resorption, which have been used in traditional Chinese medicine to treat bone disease from ancient times except for *Boerhaavia repens* L.. The result of the inhibitory activities of these drugs are shown in Table I. Thirteen MeOH extracts from *Achyranthes bidentata, B. repens, C. dahurica, C. foetida, C. heracleifolia, Cinnamomum cassia, Drynaria fortunei, Glehnia littoralis, Glycyrrhiza glabra, Lonicera japonica, Sambucus sieboldiana, Stellaria media,* and *Viscum album* var. *coloratum*. inhibited PTH-stimulated bone resorption at the concentration of 440 $\mu$g/ml, and nine of the water extracts namely, *A. bidentata, C. foetida, D. fortunei, G. glabra, Pueraria robot, S. japonica, Sparganium stoloniferum, S. media,* and *Tabanus yao* showed an inhibitory activity at the concentration of 440 $\mu$g/ml. The five MeOH extracts of *A. bidentata, B. repens, G. glabra, S.sieboldiana,* and *V. album* var. *coloratum,* and the four water extracts of *P. lobata, S. stoloniferum, S.media,* and *T. yao* showed an inibitory effect at a concentration of 44 $\mu$g/ml.

From the results of screening, the MeOH extracts of Cimicifugae Rhizoma showed strong inhibitory activities at the concentration of 440 $\mu$g/ml among the drugs used in the experiment. Therefore, the MeOH extracts of two species of *C. heracleifolia* and *C. foetida* were used for further fractionation. The hexane-, EtOAc-, *n*-BuOH-, and water-soluble fractions were assayed for bone resorption (Fig. 1). The EtOAc- and *n*-BuOH-soluble fractions showed inhibitory activiti-es on bone resorption at a concentration of 440

Table I The effect of extracts of natural crude drugs on bone resorption induced by PTH.

| Natural crude drug | Part used | Extract | Yield(%) | $^{45}$Ca release (%) | |
|---|---|---|---|---|---|
| | | | | 440 μg/ml | 44 μg/ml |
| *Achyranthes bidentata* BLUME | Root | MeOH | 7.61 | 22.36±1.69*** | 31.60±1.97*** |
| *Achyranthes bidentata* BLUME | Root | $H_2O$ | 5.68 | 48.14±1.65*** | 51.77±2.41 |
| *Boerhaavia repens* L. | Whole plant | MeOH | 8.33 | 40.30±3.83*** | 43.39±3.57* |
| *Cimicifuga dahurica* (TURCZ.) MAXIM. | Rhizoma | MeOH | 18.00 | 18.09±0.54*** | 58.85±1.89 |
| *Cimicifuga foetida* L. | Rhizoma | MeOH | 16.39 | 20.66±1.76*** | 58.33±1.97 |
| *Cimicifuga foetida* L. | Rhizoma | $H_2O$ | 13.27 | 42.71±2.75** | 56.13±2.98 |
| *Cimicifuga heracleifolia* KOMAROV | Rhizoma | MeOH | 16.76 | 16.60±0.77*** | 57.98±2.65 |
| *Cimicifuga heracleifolia* KOMAROV | Rhizoma | $H_2O$ | 19.33 | 54.78±4.15 | 49.14±3.94 |
| *Cinnamomum cassia* BLUME | Bark | MeOH | 2.89 | 28.11±1.51*** | 73.05±2.69 |
| *Cinnamomum cassia* BLUME | Bark | $H_2O$ | 2.52 | 58.21±2.34 | 62.35±1.09 |
| *Drynaria fortunei* (KUNZE) J. SMITH | Rhizoma | MeOH | 7.25 | 33.41±1.02*** | 56.45±5.75 |
| *Drynaria fortunei* (KUNZE) J. SMITH | Rhizoma | $H_2O$ | 6.34 | 48.18±3.01* | 60.44±6.25 |
| *Gentiana macrophylla* PALL. | Root | MeOH | 21.63 | 59.07±2.56 | 61.54±3.13 |
| *Gentiana macrophylla* PALL. | Root | $H_2O$ | 12.02 | 52.27±5.77 | 52.69±1.82 |
| *Glehnia littoralis* FR. SCHMIDT et MIQUEL | Root & Rhizoma | MeOH | 12.25 | 41.12±3.74* | 55.20±5.42 |
| *Glehnia littoralis* FR. SCHMIDT et MIQUEL | Root & Rhizoma | $H_2O$ | 11.28 | 69.57±3.76 | 64.24±5.67 |
| *Glycyrrhiza glabra* L. | Root & Rhizoma | MeOH | 15.35 | 18.02±0.55*** | 45.28±2.82** |
| *Glycyrrhiza glabra* L. | Root & Rhizoma | $H_2O$ | 14.67 | 44.50±4.09** | 51.63±2.47 |
| *Lonicera japonica* THUNB. | Stem & Leaves | MeOH | 7.08 | 46.47±4.12* | 50.63±3.99* |
| *Lonicera japonica* THUNB. | Stem & Leaves | $H_2O$ | 6.09 | 64.38±0.75 | 65.10±6.00 |
| *Pueraria lobata* (WILLD.) OHWI | Root | MeOH | 11.73 | 52.46±2.60 | 70.34±7.74 |
| *Pueraria lobata* (WILLD.) OHWI | Root | $H_2O$ | 19.09 | 36.18±3.91*** | 47.27±5.94* |
| *Sambucus sieboldiana* BLUME ex GRAEBN. | Stem | MeOH | 3.80 | 33.15±3.10*** | 38.02±2.44*** |
| *Sambucus sieboldiana* BLUME ex GRAEBN. | Stem | $H_2O$ | 3.53 | 66.82±3.22 | 65.81±3.22 |
| *Sophora japonica* L. | Bud | MeOH | 7.00 | 67.11±3.87 | 76.05±3.98 |
| *Sophora japonica* L. | Bud | $H_2O$ | 6.53 | 46.47±1.52** | 60.49±3.73 |
| *Sparganium stoloniferum* BUCH.-HAMIL. | Tuber | MeOH | 5.65 | 70.34±4.81 | 69.81±4.54 |
| *Sparganium stoloniferum* BUCH.-HAMIL. | Tuber | $H_2O$ | 4.79 | 46.59±2.91** | 48.05±4.12* |
| *Stellaria media* (L.) VILLARS | Whele plant | MeOH | 7.31 | 48.83±2.01* | 57.60±4.41 |
| *Stellaria media* (L.) VILLARS | Whole plant | $H_2O$ | 11.50 | 39.78±3.44*** | 46.17±4.41* |
| *Tabanus yao* MACQ. | Whole body | MeOH | 4.48 | 51.37±5.18 | 68.79±0.66 |
| *Tabanus yao* MACQ. | Whole body | $H_2O$ | 8.30 | 23.61±2.09*** | 28.72±2.96*** |
| *Viscum album* L. var *coloratum* (KOMAR.) OHWI | Stem & Leaves | MeOH | 10.94 | 26.79±0.55*** | 44.12±2.72** |
| *Viscum album* L. var *coloratum* (KOMAR.) OHWI | Stem & Leaves | $H_2O$ | 13.99 | 62.76±1.34 | 62.58±2.33 |
| PTH | | | | 59.30±2.60 | |
| Control | | | | 27.53±1.45*** | |

PTH : Bones were cultured with PTH ($2\times10^{-9}$M), n=21.
Control : Bones were cultured without PTH and extracts, n=21.
Samples : Bones were cultured with PTH ($2\times10^{-9}$M) and each extract, n=6 or 7.
Each value represents mean±S.E.
Significant decrease of calcium release compared to PTH, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

μg/ml, while the hexane- and water-soluble fractions exhibited weak inhibitory activities.

Experiments indicated that the major constiuents of EtOAc- and *n*-BuOH-soluble fractions, which were the main fractions of MeOH extract from *C. heracleifolia* and *C. foetida*, were triterpenoids. The triterpenoids were then separated by normal and reverse phase silica gel column chromatography and preparative thin layer chromatography.[13-15] Ten new triterpenoids, together with four known compounds from *C. heracleifolia* and eight new triterpenoids and three new trinor-triterpenoids, together with six known compounds from the rhizome of *C. foetida* were isolated. The chemical structuer of sixteen triterpenoids, compounds **1** : 24-*epi*-7,8-didehydrocimigenol, **2** : 2',4'-*O*-diacetyl-24-*epi*-7,8-didehydrocimigenol-3-*O*-β-xyloside, **3** : 3'-*O*-acetyl-24-*epi*-7,8-didehydrocimigenol-3-*O*-β-xyloside, **4** : 24-*epi*-7,8-

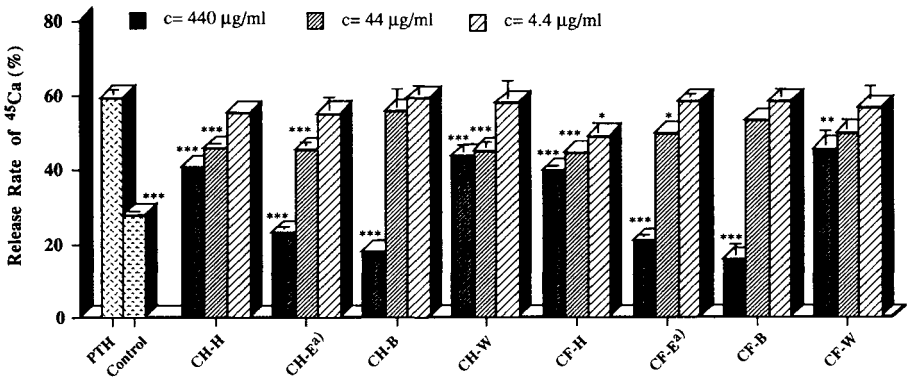


Fig. 1 Effects of fractions of MeOH extracts of rhizomes of *Cimicifuga heracleifolia* and *Cimicifuga foetida* on calcium release from bones.

CH : *Cimicifuga heracleifolia* H : Hexane fraction E : EtOAc fraction
CF : *Cimicifuga foetida* B : *n*-BuOH fraction W : Water fraction
PTH : Bone were cultured with PTH ($2\times10^{-9}$ M), n=14. Control: Bones were cultured without PTH and fractions, n=14. Samples : Bones were cultured with PTH($2\times10^{-9}$M) and each fraction, n=6 or 7. Each value represents mean±S.E. Significant decrease compared to PTH, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.
a) : EtOAc fractions of CH-E and CF-E were insoluble in medium at concentration of 440 μg/ml, so that the concentration 148 μg/ml was used for culture.

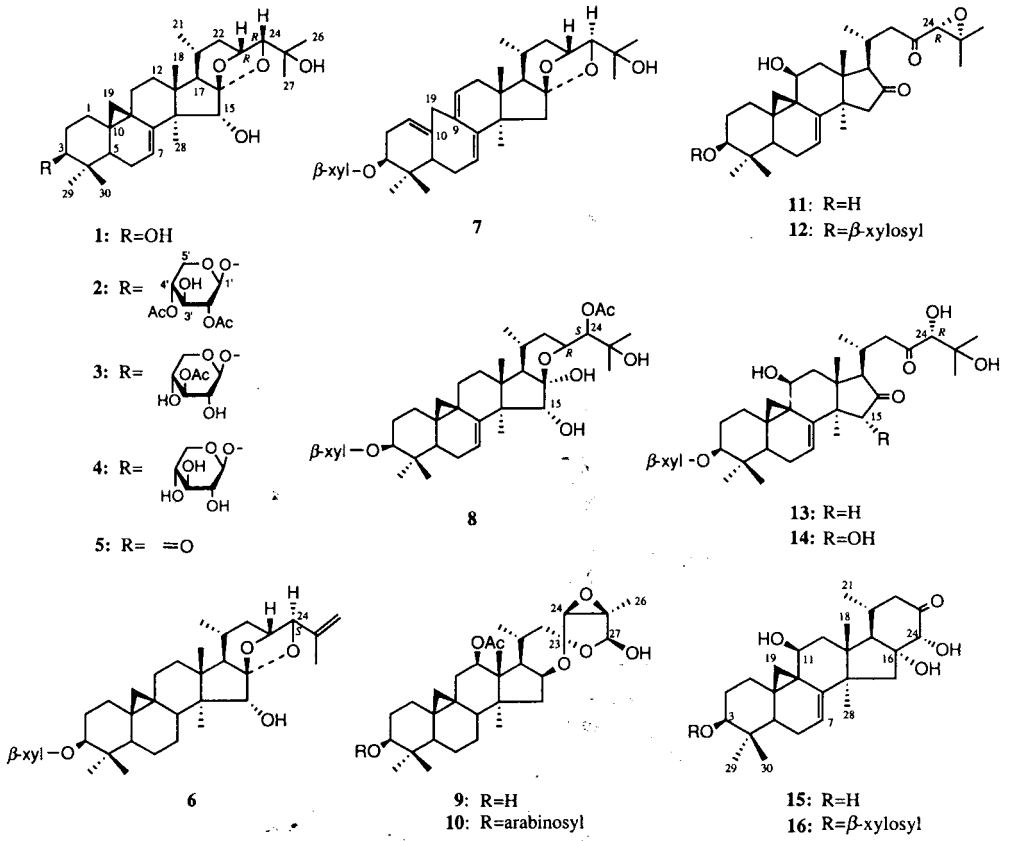


Chart 1

didehydrocimigenol-3-*O*- $\beta$-xyloside, **5**: 3-keto-24-*epi*-7,8-didehydrocimigenol and 8: 7,8-didehydro-24-*O*-acetylhydroshengmanol-3-*O*-$\beta$-xyloside from *C. heracleifolia*, while compounds **6**: 25-anhydrocimigenol-3-*O*-$\beta$-xyloside **7**: cimicinol, **9**: acetylacteol, **10**: acetylacteol-3-*O*-arabinoside, **11**: cimicidanol, **12**: cimicidanol-3-*O*-$\beta$-xyloside, **13**: cimicidol-3-*O*-$\beta$-xyloside, **14**: 15-hydroxycimicidol-3-*O*-$\beta$-xyloside, **15**: foetidinol and **16**: foetidinol-3-*O*-$\beta$-xyloside from *C. foetida*, was shown in Chart 1. These compounds were tested for their bone resorption activity. As shown in Table II, the majority of the compounds, except for compounds **1**, **5**, **11**, and **15** showed inhibitory activities on bone resorption at a concentration of 200 $\mu$M. In addition, compounds **4, 6**, and 8 exhibited suppressive effects even at a concentration of 20 $\mu$M. Triterpenoids and isoferulic acid were the main chemical component contained in Cimicifugae Rhizoma, however, isoferulic acid was found to be ineffective. From these data, the inhibitory effect of Cimicifugae Rhizoma on bone resorption is due to the triterpenoids.

The results of this study also provided a basis for structure-activity relationship analysis of triterpenoids in terms of bone resorption inhibition. It seems that cycloartenol type triterpenes, like compounds **1**, **5**, **11**, and **15,** with the exception of compound **9**, whose position-3 is hydroxy group, showed no inhibitory activity. If the hydroxy group was replaced by sugar moiety at position-3, all of these compounds showed a potent inhibitory activity. On the other hand, if the suger was acetylated, like compounds **2** and **3**, the inhibitory activity became weaker. For the side chain of triterpenoids, it seems that the acetal group may increase the inhibitory activity and 24-*epi*-7,8-didehydrocimigenol-3-*O*-xyloside (**4**) and 25-anhydrocimigenol-3-*O*-$\beta$-xyloside (**6**), which are endowed with the above mentioned functional groups, are the strongest inhibitors.

As shown in Table II, the bio-assay of individual

Table II The effect of triterpenoids on bones resorption induced by PTH.

| Compound | $^{45}$Ca release (%) | | |
|---|---|---|---|
| | 200 $\mu$M | 20 $\mu$M | 2 $\mu$M |
| 24-*epi*-7,8-Didehydrocimigenol (**1**) | 52.07±3.14 | 65.64±2.78 | 60.05±2.93 |
| 2',4'-*O*-Diacetyl-24-*epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**2**) | 42.93±1.55** | 64.58±6.65 | 63.93±5.37 |
| 3'-*O*-Acetyl-24-*epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**3**) | 32.20±4.68*** | 51.14±3.28 | 53.49±5.65 |
| 24-*epi*-7,8-Didehydrocimigenol-3-*O*-$\beta$-xyloside (**4**) | 25.82±0.88*** | 36.09±2.49*** | 52.19±3.52 |
| 3-Keto-24-*epi*-7,8-didehydrocimigenol (**5**) | 52.68±2.60 | 53.70±4.25 | 48.58±5.42 |
| 25-Anhydrocimigenol-3-*O*-$\beta$-xyloside (**6**) | 24.50±7.32*** | 37.27±1.72** | 70.12±5.88 |
| Cimicinol (**7**) | 29.22±1.46*** | 51.18±3.15 | 60.16±7.32 |
| 7,8-Didehydro-24-*O*-acetylhydroshengmanol-3-*O*-$\beta$-xyloside (**8**) | 35.99±4.68** | 40.28±3.28** | 59.90±6.56 |
| Acetylacteol (**9**) | 18.14±0.71*** | 57.69±4.69 | 57.14±3.78 |
| Acetylacteol-3-*O*-arabinoside (**10**) | 17.13±0.36*** | 62.78±1.24 | 69.75±5.48 |
| Cimicidanol (**11**) | 68.81±4.10 | 69.53±4.20 | 65.77±3.76 |
| Cimicidanol-3-*O*-$\beta$-xyloside (**12**) | 22.69±0.88** | 52.98±2.93 | 56.84±4.75 |
| Cimicidol-3-*O*-$\beta$-xyloside (**13**) | 47.24±1.62* | 54.71±3.18 | 55.78±5.06 |
| 15 $\alpha$-Hydroxycimicidol-3-*O*-$\beta$-xyloside (**14**) | 44.68±3.18* | 47.38±4.75 | 55.96±4.54 |
| Foetidinol (**15**) | 53.21±1.26 | 60.08±1.39 | 64.76±2.10 |
| Foetidinol-3-*O*-$\beta$-xyloside (**16**) | 41.99±3.15** | 58.47±3.34 | 55.42±3.40 |
| Isoferulic acid | 56.40±3.71 | 61.08±3.52 | 58.98±2.97 |
| Ipriflavone | 36.06±2.95*** | 45.41±1.79* | 57.27±5.37 |
| PTH | | 59.54±3.92 | |
| Control | | 30.19±2.02*** | |

PTH : Bones were cultured with PTH ($2\times10^{-9}$M), n=14.
Control : Bones were cultured without PTH and compounds, n=14
Samples : Bones were cultured with PTH ($2\times10^{-9}$M) and each compound, n=6 or 7.
Each value represents mean±S.E.
Significant decrease of calcium release compared to PTH, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

compound was carried out, however, in some cases, slight or non activity was found when they were tested individually. The compounds **4** and **8** showed inhibitory activity at 20 $\mu$M and compounds **2**, **10**, **12**, and **13** exhibited no activity at the same concentration when they were assayed individually. When natural products show slight or no activity, they are further assayed by making a mixture as a synergistic effect might operate among them.[19] Based on this ground, we prepared two mixtures, mixture A (containing triterpenoids **2**, **4**, and **8** as they were main constituents of *C. heracleifolia*) and mixture B (containing **10**, **12** and **13** as they were main constituents of *C. foetida*), and their activity was checked by *in vitro* assay. Also a comparative study was made of mixture A and B with ipriflavone which is commercially available useful drug for osteoporosis, towards the inhibitory activity on bone resorption. It was found that the inhibitory activity of these two mixtures at 66.67 $\mu$M were stronger than that of ipriflavone at 200 $\mu$M (Table II and Fig. 2).[20]

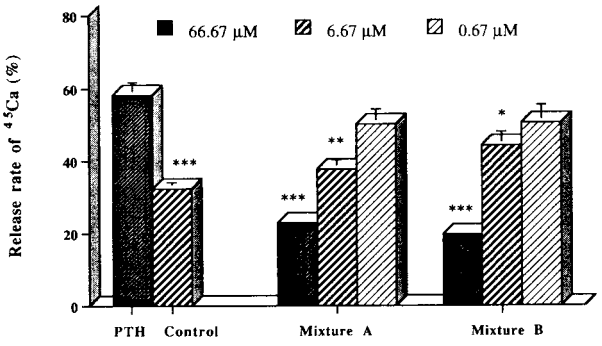


Fig. 2 Effects of mixtures of triterpenoids on calcium release from bones.
PTH : Bones were cultured with PTH ($2\times10^{-9}$M), n=14. Control : Bones were cultured without PTH and fractions, n=14. Mixture A: Mixture of triterpenoids **2**, **4**, and **8** (1 : 1 : 1), n=6 or 7. Mixture B : Mixture of triterpenoids **10, 12**, and **13** (1 : 1 : 1), n=6 or 7. Each value represents mean± S.E. Significant decrease compared to PTH, $*p<0.05$, $**p<0.01$, $***p<0.001$.

Table III Recovery of PTH-stimulated bone resorption from inhibition by EtOAc-soluble fractions and triterpenoids

| Sample | Concentration | $^{45}$Ca release (%) | |
|---|---|---|---|
| | | 0-72 hrs | 73-144 hrs |
| PTH | 2 nM | 26.10±1.45 | 59.90±1.90 |
| CH-E[a] | 147 $\mu$g/ml | 13.01±0.35[c] | 24.25±0.93[c] |
| CH-E[b] | 147 $\mu$g/ml | 14.60±0.95[c] | 39.89±1.87[f] |
| CH-E[a] | 44 $\mu$g/ml | 25.17±0.75 | 43.47±1.41[d] |
| CH-E[b] | 44 $\mu$g/ml | 21.87±2.67 | 59.29±3.45 |
| CF-E[a] | 147 $\mu$g/ml | 15.10±1.40[c] | 19.39±0.71[c] |
| CF-E[b] | 147 $\mu$g/ml | 18.74±1.40[d] | 58.84±3.03 |
| CF-E[a] | 44 $\mu$g/ml | 29.20±0.66 | 48.30±1.25[d] |
| CF-E[b] | 44 $\mu$g/ml | 26.64±3.09 | 63.65±3.01 |
| 2',4'-*O*-Diacetyl-24-*epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**2**)[a] | 200 $\mu$M | 23.90±1.85 | 44.38±1.28[d] |
| 2',4'-*O*-Diacetyl-24-*epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**2**)[b] | 200 $\mu$M | 24.90±2.88 | 71.88±2.24 |
| 24-*Epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**4**)[a] | 200 $\mu$M | 17.92±0.63[d] | 26.02±1.01[e] |
| 24-*Epi*-7,8-didehydrocimigenol-3-*O*-$\beta$-xyloside (**4**)[b] | 200 $\mu$M | 18.58±1.26[d] | 56.06±1.54 |
| Cimicidanol-3-*O*-$\beta$-xyloside (**12**)[a] | 200 $\mu$M | 16.37±0.66[d] | 22.70±0.87[e] |
| Cimicidanol-3-*O*-$\beta$-xyloside (**12**)[b] | 200 $\mu$M | 17.79±0.78[d] | 56.62±3.80 |
| Cimicidol-3-*O*-$\beta$-xyloside (**13**)[a] | 200 $\mu$M | 20.54±1.14[c] | 44.17±2.26[d] |
| Cimicidol-3-*O*-$\beta$-xyloside (**13**)[b] | 200 $\mu$M | 17.93±0.52[d] | 58.25±3.03 |

CH-E : EtOAc-soluble fraction of MeOH extract from *C.heracleifolia*. CF-E : EtOAc-soluble fraction of MeOH extract from *C. foetida*.
a) Bones were cultured with PTH and samples for 144 hrs. b) Bones were cultured with PTH and samples for 72 hrs and after 72 hrs, bones were cultured with PTH only.
Values are expressed as mean±S.E., n=6 or 7. Significant effects of samples, c, $p<0.05$, d, $p<0.01$. e, $p<0.001$. Significant effect of sample removal, f, $p<0.001$.

Interestingly, as expected, mixture A at 6.67 $\mu$M exhibited more potent inhibitory activity than that of ipriflavone at 20 $\mu$M, whereas mixture B at 6.67 $\mu$M showed the same inhibitory activity as ipriflavone at 20 $\mu$M. It is suggested that a synergistic effect should exist among these compounds. Furthermore as mentioned above, no activity was found in the case of compound **10**, **12**, and **13** at 20 $\mu$M assaying individually, it is pertinent to think that a more potent synergistic effect might exist among these compounds in mixture B as they showed significant activity. However, further research is needed to elucidate the machanism of their synergistic effect.

For the toxicity of these samples, the recovery experiment was performed.[21] As shown in Table III, in PTH-stimulated cultures, the inhibitory effects of EtOAc-soluble fraction of MeOH extract from *C. foetida* abbreviated by CF-E at concentration of 44, 147 $\mu$g/ml, compounds **2**, **4**, **12**, **14** at 200 $\mu$M were reversed after removal of the above tested samples. Partial recovery was obtained by removing 147 $\mu$g/ml of EtOAc-soluble fraction of MeOH extract from *C. heracleifolia* abbreviated by CH-E, but its effect was reversible at submaximal concentration (44 $\mu$g/ml). These results clearly indicate that in PTH-stimulated cultures these samples did not possess irreversible toxicity.

So far, most of the investigation on Cimicifugae Rhizoma were focused on its anti-inflammatory activities.[22-24] This is the first time a potent inhibitory activity of this drug on bone resorption induced by PTH has been found. It is possible that the inhibitor of PTH-stimulated bone resorption may be used to treat osteoporosis. There has not been enough study on natural products with anti-osteoporosis activity from plants up to now. Our findings suggest the Cimicifugae Rhizoma has a potential to passibly be an anti-osteoporosis agent. The mechanism of inhibitory action and anti-osteoporosis *in vivo* are now under investigation in our laboratory.

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## 和文抄録

副甲状腺ホルモン（PTH）による骨吸収亢進器官培養系を用い，34種の生薬のメタノールと水エキスについて骨吸収亢進に対する抑制作用を検討し，13種のメタノールと9種の水エキスに有意な抑制効果を認めた。これらの生薬のうち，強い活性が認められた関升麻（*Cimicifuga heracleifolia* KOMAROV）および川升麻（*Cimicifuga foetida* L.）のメタノールエキスをさらにヘキサン，酢酸エチル，ブタノール，水可溶部に分画し，その活性を比較検討したところ，酢酸エチルとブタノール可溶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17種の化合物についても同様な方法で検討を行った結果，サイクロアルテノール型のトリテルペノイド類が升麻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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