# Effects on Cultured Neonatal Mouse Calvaria of the Flavonoids Isolated from *Boerhaavia repens*

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A MeOH extract from the whole plant of *Boerhaavia repens* was found to inhibit bone resorption induced by parathyroid hormone (PTH) in tissue culture. Systematic separation of the MeOH extract afforded one new and two known flavonoid glycosides, namely, eupalitin 3-*O*-*β*-D-galactopyranosyl-(1→2)-*β*-D-glucopyranoside (**1**), eupalitin 3-*O*-*β*-D-galactopyranoside (**2**), and 6-methoxykaempferol 3-*O*-*β*-D-(1→6)-robinoside (**3**). The structure of the new compound **1** was determined using spectroscopic techniques. The inhibitory activity of these substances toward bone resorption induced by PTH was evaluated, and compounds **1** and **2** were found to exhibit significant activity.

Osteoporosis, which has been defined as a "state of low bone mass", is one of the major problems in our aging society. Osteoporosis results in bone fracture in older members of the population, especially in post-menopausal women.[1] It is well known that parathyroid hormone (PTH) plays an important role in the regulation of the calcium metabolism of bone and is widely used as a bone resorption stimulator in bone-tissue-culture systems designed to evaluate the direct effects of compounds under test.[2,3] In traditional medicine, there are many natural crude drugs that have the potential for use to treat bone diseases; however, not much laboratory work has been reported evaluating this possible use. One example, however, is ipriflavone, a natural product derivative, has been used clinically for this purpose.[4,5]

In a search for natural crude drugs having inhibitory activity on bone resorption, we have screened a number of plants widely used in traditional medicine for their inhibitory activity on bone resorption induced by PTH in organ culture.[6] Among these crude drugs, a MeOH extract of *Boerhaavia repens* L. (Nyctaginaceae) was found to be active and has been targeted for the present study. This plant is distributed in tropical and subtropical regions of Asia, Africa, and America and has wide use in traditional medicine. For example, the roots of *B. repens* can ease back pain and also have been used as a laxative, diuretic, and emetic. Moreover, *B. repens* roots have been reported to be used in treating asthma and abdominal tumors.[7] In the present work, bioactivity-guided fractionation of a MeOH extract of *B. repens* and purification of the active constituents led to the isolation of a new flavonoid glycoside (**1**) together with two known flavonoid glycosides (**2** and **3**). In this work we report the isolation and characterization of the isolated flavonoid glycosides and their inhibitory effects on bone resorption induced by PTH in bone organ culture.

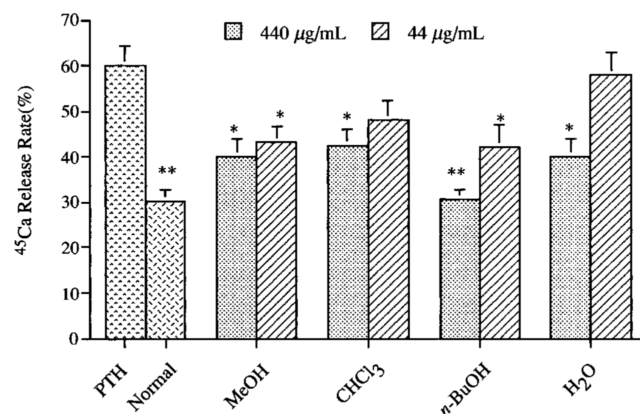


**Figure 1.** $^{45}$Ca-release inhibitory activity of various fractions of *Boerhaavia repens*. PTH: Bones were cultured with PTH ($2 \times 10^{-9}$ M). Normal: Bones were cultured without PTH and fractions. Samples: Bones were cultured with PTH ($2 \times 10^{-9}$ M) and each fraction. Each value represents the mean ± SE, $n = 6$ or 7. Significant decrease compared to PTH group, $*p < 0.05$, $**p < 0.001$.

## Results and Discussion

As indicated above, after screening 18 natural crude drugs for their inhibitory activity on bone resorption induced by PTH in tissue culture,[6] a MeOH extract of *B. repens* was found to be the most potent inhibitor of bone resorption at a concentration of 440 μg/mL and was further fractionationed into $CHCl_3$-, *n*-BuOH-, and $H_2O$-soluble fractions. These fractions were assayed for bone resorption. As can be seen in Figure 1, the *n*-BuOH-soluble fraction showed a significant inhibitory activity on PTH-stimulated bone resorption at a concentration of 440 μg/mL, while the $CHCl_3$- and $H_2O$-soluble frac-

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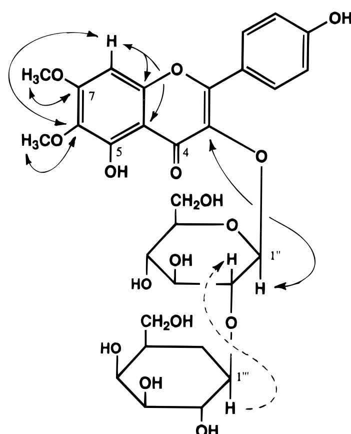



**Figure 2.** HMBC (solid arrow) and NOE (dashed arrow) correlations of compound **1**.

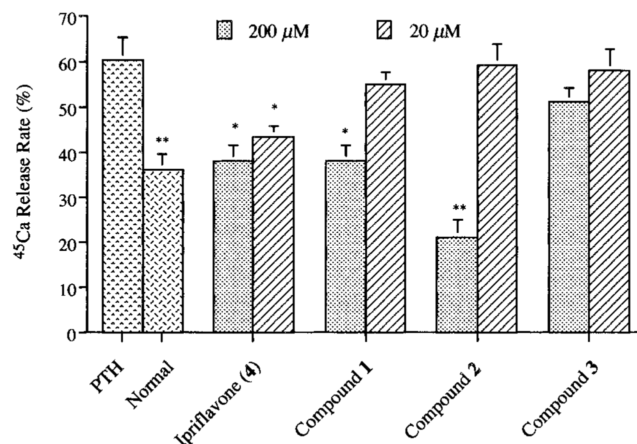


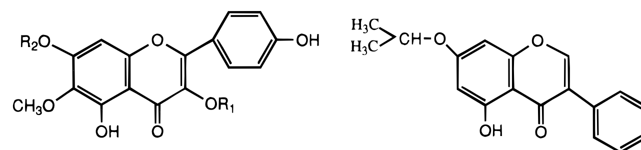

**Figure 3.** $^{45}$Ca-release inhibitory activity of compounds **1**–**3** compared with ipriflanove (**4**). PTH: Bones were cultured with PTH ($2 \times 10^{-9}$ M). Normal: Bones were cultured without PTH and test compounds. Samples: Bones were cultured with PTH ($2 \times 10^{-9}$ M) and each compound. Each value represents the mean ± SE, $n = 6$ or 7. Significant decrease compared to PTH group, $^{*}p < 0.05$, $^{**}p < 0.001$.

tions exhibited weak inhibitory activities. Therefore, the *n*-BuOH-soluble fraction was subjected to further chemical analysis and, after column chromatographic separation followed by preparative TLC, three compounds (**1**–**3**) were isolated.

Eupalitin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside (**1**), isolated as a yellow powder, showed an $[\alpha]_D$ −19.2° (*c* 0.3, MeOH). The positive-ion FABMS and HRFABMS showed an ion peak $[M + H]^+$ at $m/z$ 655 and 655.1921 (calcd 655.1874), indicating a molecular formula of $C_{29}H_{34}O_{17}$. The IR spectrum revealed absorptions at 3350, 2900, 1650, 1590 $cm^{-1}$. The $^1$H- and $^{13}$C-NMR spectra suggested that **1** was a flavone type of compound and contained some signals that were assignable to glucose and galactose from the detailed analysis of the $^1$H- and $^{13}$C-NMR spectra. In the $^1$H-NMR spectrum, the presence of only one proton at δ 6.60 (1H, s, H-8) indicated that the A-ring was trisubstituted. Furthermore, two sets of *ortho*-coupled aromatic proton signals (δ 7.37, 2H, d, $J = 8.8$ Hz, H-3′, H-5′; 8.57, 2H, d, $J = 8.8$ Hz, H-2′, H-6′) were apparent in the $^1$H-NMR spectrum, suggesting that the 4′-position of the B-ring was substituted. To clarify the position of the methoxy groups (δ 3.85, 3.98), an HMBC NMR spectrum (Figure 2) of **1** was run, and the positions of the two methoxy groups were confirmed at C-6 and C-7. Thus, the aglycon of the molecule of **1** was identified as eupalitin.[9] Next, the positions of the glucose and galactose moieties were determined by HMBC and NOE NMR experiments. The anomeric proton of glucose at δ 5.50 showed long-range correlations with C-3 (δ 135.0) of the aglycon moiety, indicating that glucose was connected directly to the aglycon. Some other significant long-range correlations are shown by arrows in Figure 2. Also, on irradiation of H-1‴ (δ 6.70), an increase in the intensity of H-2″ (δ 4.21) occurred, suggesting that the anomeric carbon (position 1‴) of galactose was connected to the C-2″ of the glucose unit (Figure 2). On the basis of the foregoing evidence, the structure of compound **1** was concluded to be eupalitin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside (**1**).

The other two flavonoid glycosides, eupalitin 3-*O*-β-D-galactopyranoside (**2**) and 6-methoxykaempferol 3-*O*-β-D-(1→6)-robinoside (**3**) have already been reported from *Ageratina calophylla* [9] and *Brickellia arguta*,[10] respectively. This is the first report, however, in which all the $^{13}$C-NMR signals of **2** and **3** have been assigned, and these assignments were based on NOE, DEPT, $^1$H–$^1$H COSY, $^1$H–$^{13}$C COSY, and HMBC experiments.

The three flavonoid glycosides (**1**–**3**) were tested for their bone resorption activity. As shown in Figure 3, two compounds (**1** and **2**) showed inhibitory activity on PTH-stimulated bone resorption at a concentration of 200 μM; however, compound **3** was found to be inactive at the same concentration. Ipriflavone (**4**), a clinically used natural product derivative in Japan and Italy,[11] is reported to have a suppressive effect on PTH-stimulated bone resorption in a rat long-bone culture system.[12] Thus, the inhibitory activity of compounds **1** and **2** was further compared with ipriflavone (**4**) and found to exhibit stronger activity at a concentration of 200 μM (Figure 3).




**1:** $R_1$ = β-D-gal $\xrightarrow{2}$ β-D-glc, $R_2 = CH_3$
**2:** $R_1$ = β-D-gal, $R_2 = CH_3$
**3:** $R_1$ = β-D-(1→6)-robinobiose, $R_2$ = H

**4**

It is pertinent to consider that the inhibitory activity of drugs is sometimes a result of their toxic effects, and consequently, an erroneous conclusion might occur in screening studies like the present case. Thus, to avoid such confusion, a recovery experiment was performed.[13] As shown in Table 1, the suppressive effect on bone resorption by compounds **1** and **2** at maximal concentration (200 μM) could be reversed by removing these compounds. These results indicate that in PTH-stimulated bone culture, the inhibitory effects of compounds **1** and **2** did not appear to cause irreversible toxicity.

Finally, we have attempted to better understand the structure–activity relationship of these flavone glycosides on bone resorption induced by PTH. As shown in Figure 3, compounds **1** and **2**, bearing a methoxy group at position C-7, are potent inhibitors of bone resorption, although compound **3**, with a C-7 OH group, showed no

**Table 1.** Recovery of PTH-Stimulated Bone Resorption from Inhibition by Compounds **1** and **2**[a]

| sample | % $^{45}$Ca release | |
|---|---|---|
| | 0–3 days | 4–6 days |
| PTH | 28.7 ± 2.3 | 61.8 ± 2.7 |
| control | 20.0 ± 2.8 | 37.3 ± 4.3 |
| **1** (200 μM, 0–6 days) | 29.1 ± 2.1 | 38.0 ± 0.9 * [b] |
| **1** (0–3 days), PTH (4–6 days) | 29.0 ± 2.2 | 60.3 ± 4.0 |
| **2** (200 μM, 0–6 days) | 20.3 ± 1.6 | 23.1 ± 1.9 ** [b] |
| **2** (0–3 days), PTH (4–6 days) | 20.6 ± 1.5 | 55.3 ± 5.4 |

[a] Values are the mean ± SE, $n$ = 6 or 7. [b] Significant effects of compounds, $*p < 0.05$, $**p < 0.001$.

inhibitory activity. However, the type of sugar moiety at position C-3 in these compounds seems to have no effects on activity. Further studies on the inhibitory activity of various related flavonoid glycosides could provide further structure–activity relationship information. Our experimental work is mainly based on in vitro experiments, but further in vivo experiments are in progress. The inhibitory mechanism of action of these compounds is still unknown, but is being investigated in our laboratory.

## Experimental Section

**General Experimental Procedures.** IR spectra were recorded on a Hitachi 260-01 spectrometer using KBr disks. UV spectra were taken on a Shimadzu UV-2200 UV-vis spectrophotometer. Optical rotations were measured in MeOH solutions on a JASCO DIP-360 digital polarimeter at 25 °C. $^1$H- and $^{13}$C-NMR spectra were taken on a JNM-GX 400 spectrometer in pyridine-$d_5$ with $Me_4Si$ as an internal standard, and chemical shifts are recorded as $\delta$ values. $^1$H–$^1$H COSY, $^1$H–$^{13}$C COSY, and HMBC NMR spectra were obtained with the usual pulse sequences, and data processing was performed with standard JEOL software. FABMS and HRFABMS data were obtained with a JEOL JMS 102A spectrometer (ionization voltage, 70 eV; accelerating voltage, 5.0 kV) using glycerol + *m*-nitrobenzyl alcohol as the matrix. Column chromatography was performed with Wakogel C-200 or Cosmosil 140 $C_{18}$ column. TLC and preparative TLC were carried out on precoated Kieselgel $F_{254}$ plates (0.25 or 0.5 mm) or Merck RP-18 $F_{254}$ reversed-phase plates (0.25 mm) developed with EtOAc–acetone–$H_2O$ (6:4:1), and spots were detected under a UV light or by spraying with $Ce(SO_4)_2$–10% $H_2SO_4$ (1:99).

**Plant Material.** *B. repens* was collected at Malir (Karachi, Pakistan) in October 1993. This sample was identified by Prof. Dr. M. Qaiser (Department of Botany, University of Karachi), and a voucher sample was deposited at the Museum of Materia Medica of Toyama Medical and Pharmaceutical University, Toyama, Japan.

**Extraction and Isolation.** The shade-dried whole plant (1.2 kg) was chopped into small pieces and refluxed with MeOH for 3 h (900 mL × 2). The total filtrate was evaporated under reduced pressure to obtain a dark green mass, and this extract was suspended in distilled $H_2O$ and partitioned with $CHCl_3$ and *n*-BuOH successively. The *n*-BuOH fraction showed strong inhibitory activity against bone resorption; thus, 1.2 g of the *n*-BuOH fraction was purified repeatedly by preparative TLC using EtOAc–acetone–$H_2O$ (6:4:1) as solvent to yield eupalitin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranoside (**1**) (7.4 mg), eupalitin 3-*O*-β-D-galactopyranoside (**2**) (14.4 mg), and 6-methoxykaempferol 3-*O*-β-D-(1→6)-robinoside (**3**) (7.4 mg).

**Eupalitin 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (1):** a yellow amorphous residue; $[\alpha]_D$ −19.2° ($c$ 0.3, MeOH); IR $\nu$ max 3350 (OH), 2900, 2200, 1650 (C=O), 1590, 1440 cm$^{-1}$; $^1$H NMR (pyridine-$d_5$) $\delta$ 3.85 (3H, s, MeO-7), 3.98 (3H, s, MeO-6), 6.60 (1H, s, H-8), 7.37 (2H, d, $J$ = 8.8 Hz, H-3′, H-5′), 8.57 (2H, d, $J$ = 8.8 Hz, H-2′, H-6′); glucosyl, 3.84 (1H, m, H-5), 4.21 (1H, dd, $J$ = 7.0, 5.0 Hz, H-2), 4.22 (1H, m, H-3), 4.23 (1H, m, H-4), 4.32 (1H, dd, $J$ = 12.0, 3.0 Hz, H-6), 4.43 (1H, dd, $J$ = 12.0, 3.0 Hz, H-6), 5.50 (1H, d, $J$ = 7.0 Hz, H-1); galactosyl, 4.14 (1H, br t, $J$ = 6.0 Hz, H-5), 4.23 (1H, dd, $J$ = 11.0, 6.0 Hz, H-6), 4.34 (1H, dd, $J$ = 11.0, 6.0 Hz, H-6), 4.39 (1H, dd, $J$ = 9.0, 3.0 Hz, H-3), 4.59 (1H, br d, $J$ = 3.0 Hz, H-4), 4.93 (1H, dd, $J$ = 9.0, 7.5 Hz, H-2), 6.70 (1H, d, $J$ = 7.5 Hz, H-1); $^{13}$C NMR (pyridine-$d_5$) $\delta$ 56.4 (q, MeO-7), 60.9 (q, MeO-6), 91.1 (d, C-8), 106.6 (s, C-10), 116.3 (d, C-3′, C-5′), 122.2 (s, C-1′), 132.0 (d, C-2′, C-6′), 134.3 (s, C-6), 135.0 (s, C-3), 152.3 (s, C-9), 153.0 (s, C-5), 156.9 (s, C-2), 159.1 (s, C-7), 161.7 (s, C-4′), 179.0 (s, C-4); glucosyl, 62.5 (t, C-6), 71.2 (d, C-4), 77.7 (d, C-3), 78.6 (d, C-5), 82.7 (d, C-2), 100.1 (d, C-1); galactosyl, 61.5 (t, C-6), 69.4 (d, C-4), 75.4 (d, C-2), 76.1 (d, C-3), 78.4 (d, C-5), 106.3 (d, C-1); positive-ion FABMS $m/z$ $[M + H]^+$ 655; HRFABMS $m/z$ $[M + H]^+$ 655.1921 (calcd for $C_{29}H_{35}O_{17}$, 655.1874).

**Eupalitin 3-*O*-β-D-galactopyranoside (2)**[9]: yellow amorphous residue; $[\alpha]_D$ −24.0° ($c$ 0.1, MeOH); IR $\nu$ max 3400 (OH), 2900, 2200, 1600 (C=O), 1507, 1440 cm$^{-1}$; $^{13}$C NMR (pyridine-$d_5$) $\delta$ 56.4 (q, MeO-7), 60.5 (q, MeO-6), 91.8 (d, C-8), 106.5 (d, C-10), 116.1 (d, C-3′, C-5′), 121.9 (s, C-1′), 131.9 (d, C-2′, C-6′), 132.7 (s, C-6), 135.0 (s, C-3), 152.6 (s, C-9), 153.0 (s, C-5), 157.7 (s, C-2), 159.3 (s, C-7), 161.7 (s, C-4′), 179.0 (s, C-4); galactosyl, 61.8 (t, C-6), 69.7 (d, C-4), 73.2 (d, C-2), 75.2 (d, C-3), 77.5 (d, C-5), 104.2 (d, C-1); positive-ion FABMS $m/z$ $[M + H]^+$ 493; HRFABMS $m/z$ $[M + H]^+$ 493.1335 (calcd for $C_{23}H_{25}O_{12}$, 493.1346).

**6-Methoxykaempferol 3-*O*-β-D-(1→6)-rhamnoside (3)**[10]: yellow amorphous residue; $[\alpha]_D$ −30.6° ($c$ 0.1, MeOH); IR $\nu$ max 3400 (OH), 2900, 2200, 1600 (C=O), 1507, 1440 cm$^{-1}$; $^{13}$C NMR (pyridine-$d_5$) $\delta$ 60.2 (q, MeO-6), 94.9 (d, C-8), 105.1 (s, C-10), 116.0 (d, C-3′, C-5′), 121.9 (s, C-1′), 132.0 (d, C-2′, C-6′), 132.4 (s, C-6), 135.0 (s, C-3), 153.0 (s, C-9), 153.5 (s, C-5), 158.0 (s, C-2), 159.0 (s, C-7), 161.7 (s, C-4′), 179.0 (s, C-4); galactosyl, 67.1 (t, C-6), 69.7 (d, C-4), 73.1 (d, C-2), 75.2 (d, C-5), 75.2 (d, C-3), 105.1 (d, C-1); rhamnosyl, 18.5 (q, C-6), 69.7 (d, C-4), 72.1 (d, C-2), 72.6 (d, C-3), 73.8 (d, C-5), 102.0 (d, C-1); positive-ion FABMS $m/z$ $[M + H]^+$ 625; HRFABMS $m/z$ $[M + H]^+$ 625.1774 (calcd for $C_{28}H_{33}O_{16}$, 625.1768).

**Bone Organ Culture System.** An assay to evaluate bone-resorbing activity as reported by Shigeno *et al.* [8] was used. Briefly, 2-day-old mice were injected subcutaneously with $^{45}CaCl_2$ (2 μCi). Two days later, the parietal bones were taken out and cultured in sterile plastic multiwell culture plates (well area, 2 cm$^2$) on stainless steel grids in the top of glass rings that supported the bones near the gas–liquid interface. Ham's F-12 medium (1 mL/well), which consisted of 10.6 g/L Ham's F-12 medium, 2.2 g/L $NaHCO_3$, 1.0 mM $CaCl_2$, and 5% (v/v) heat-inactivated horse serum, was

used. Bones were incubated in an incubator at 37 °C under 5% $CO_2$ in air. Bones were randomly assigned to control and treated groups and each group consisted of six or seven bones. After preculturing for 24 h, the medium was removed and fresh medium containing PTH (final concentration, $2 \times 10^{-9}$ M) and the samples to be tested were added, then the bones were incubated for 144 h. In each experimental period, after 72 h, the medium was changed with fresh medium. After finishing the culture, bones were removed and put in 0.01 M EDTA-acetate buffer solution (pH 5.5) to extract $^{45}Ca$ contained in bone. $^{45}Ca$ released into the culture medium from prelabeled bones at 72 h and 144 h and the EDTA solution were counted separately. Bone resorption was assessed as the percentage of total $^{45}Ca$ that was released into the medium during the culture. $^{45}Ca$ was measured by liquid scintillation counting.

All of the tested samples, except for the aqueous fraction, were dissolved in DMSO and added at a DMSO concentration of 0.1%. DMSO at 0.1% was added to normal, PTH, and aqueous fraction groups and showed no effect on bone resorption.

**Recovery Assay.** A recovery assay was performed as described above. Bones alone were cultured with PTH and compounds for 72 h. After 72 h, compounds were removed and bones were cultured with PTH only.

**Statistical Analysis.** Significance levels of the mean differences in the bone resorption assay were analyzed by Student's *t*-test, and $p$ values $< 0.05$ were considered as significant.

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## References and Notes

(1) McGowan, J. A. *Aging* **1993**, *5*, 81−93.
(2) Dempster, D. W.; Cosman, F.; Parisien, M.; Shen, V.; Lindsay, R. *Endocrine Rev.* **1993**, *14*, 690−709.
(3) Okamoto, Y.; Ota, K. *Jpn. J. Clin. Med.* **1994**, *675*, 2295−2300.
(4) Fujita, T.; Yoshikawa, S.; Ono, K.; Inoue, T.; Orimo, H. *J. Clin. Exp. Med.* **1986**, *138*, 113−141.
(5) Passeri, M.; Biondi, M.; Costi, D.; Bufalino, L.; Castiglione, G. N.; Di Peppe, C.; Abate, G. *Bone Miner.* **1992**, *19 (Suppl. 1)*, S57−62.
(6) Li, J. X.; Kadota, S.; Li, H. Y.; Miyahara, T.; Namba, T. *J. Trad. Med.* **1996**,*13*, 50−58.
(7) Namba, T. *Medicinal Resources and Ethnopharmacology in Sri Lanka and Nepal, Studia Bonorum Materierum Medica No. 1*, Toyama Medical and Pharmaceutical University: Toyama, Japan, 1983; pp 20, 277, 279.
(8) Shigeno, C.; Yamamoto, I.; Dokoh, S.; Hino, M.; Aoki, J.; Yamada, K.; Morita, R.; Kameyama, M.; Torizuka, M. *J. Clin. Endocrin. Metab.* **1985**, *61*, 761−768.
(9) Fang, N.; Yu, S.; Mabry, T. J. *Phytochemistry* **1986**, *25*, 2684−2686.
(10) Rosler, K. A.; Goodwin, R. S.; Mabry, T. J.; Varma, S. D.; Norris, J. *J. Nat. Prod.* **1984**, *47*, 316−319.
(11) Tsunenari, T.; Tsutsumi, M. *J. Clin. Exp. Med.* **1993**, *165*, 655−657.
(12) Tsuda, M.; Kitazawa, T.; Ito, T.; Fujita, T. *J. Bone Miner. Res.* **1986**, *1*, 207−211.
(13) Klein-Nulend, J.; Raisz, L. R. *Endocrinology* **1989**, *125*, 1019−1024.

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