

SHORT COMMUNICATION

Stimulatory Constituents of *Eclipta prostrata* on Mouse Osteoblast Differentiation

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One flavonoid, diosmetin (1), and two isoflavonoids, 3'-hydroxybiochanin A (2) and 3'-O-methylroboflavone (3), were isolated from the methanol extract of *Eclipta prostrata* L. by a bioactivity-guided fractionation technique using primary cultures of mouse osteoblasts as an *in vitro* assay system. All three compounds significantly increased osteoblast differentiation as assessed by the alkaline phosphatase activity. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: *Eclipta prostrata*; Compositae; isoflavonoid; osteoblast differentiation; alkaline phosphatase.

INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by a reduction in bone mass and deterioration in bone architecture, resulting in increased bone fragility and its susceptibility to fractures (Rodan and Martin, 2000). Osteoporosis is known to be caused by a relative increase of bone resorption over bone formation. In bone formation, osteoblasts play a crucial role through the proliferation and differentiation (Rodan and Martin, 2000). During differentiation, osteoblasts produce almost all of the constituents of the bone matrix and direct its subsequent mineralization (Manolagas, 2000). Therefore, stimulation of osteoblast differentiation has been suggested to be an important therapeutic approach for the prevention and/or treatment of osteoporosis.

To find natural products having a stimulatory activity on osteoblast differentiation, several hundreds of plant extracts were screened employing primary cultures of mouse calvarial osteoblasts as an *in vitro* assay system. In the course of screening, a methanol extract of the aerial parts of *Eclipta prostrata* L. (Compositae) was found significantly to increase osteoblast differentiation as assessed by alkaline phosphatase (ALP) activity, one of the major osteoblast differentiation markers. Thiophenes, triterpenes, coumestanes and flavonoids have been reported as constituents of *Eclipta* species (Yahara *et al.*, 1997; Singh and Bhargava, 1992). To date, however, there have been no previous studies of *E. prostrata* on the stimulation of osteoblast differentiation. Hence, this study attempted to isolate compounds having stimulatory activity on osteoblast differentiation from the aerial parts of *E. prostrata*.

MATERIAL AND METHODS

General experimental procedures. The ^1H and ^{13}C NMR measurements were carried out in a Bruker AMX 400 spectrometer operating at 300 and 100 MHz, respectively. Solvent signals were used as internal standards. ^1H - ^1H COSY, HMQC and HMBC NMR experiments were performed on the same spectrometer. EI-mass spectra were obtained on a VG Trio 2 spectrometer with a 70 eV ionizing potential. TLC and column chromatography (CC) were carried out on precoated silica gel F₂₅₄ plates (art. 5715, Merck), RP-18 F₂₅₄ plates (art. 15423, Merck), silica gel 60 (230–400 mesh, Merck) and Sephadex LH 20 (18–110 μm , Pharmacia Co. Ltd).

Plant material. The aerial parts of *E. prostrata* were purchased from Kyung-dong Market, Seoul, Korea in June 2004, and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University. A voucher specimen (SNUPH-EP2004-06) has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and isolation. The aerial parts of *E. prostrata* (9 kg) were extracted three times with 80% MeOH to obtain a methanol extract (831 g). The methanol extract was suspended in H₂O and partitioned successively with *n*-hexane, CHCl₃, EtOAc and *n*-BuOH. The CHCl₃ fraction (77 g) was subjected to CC over silica gel and eluted with a *n*-hexane–EtOAc step gradient to give 11 fractions (Fr. 1 to Fr. 11). Fraction 10 was subjected to CC over silica gel eluted with a *n*-hexane–EtOAc step gradient to afford 15 fractions (Fr. 10-1 to Fr. 10-15). Fraction 10-13 was applied to CC over Sephadex LH-20 eluted with a *n*-hexane–CH₂Cl₂–MeOH (5:5:1) mixture to give four fractions (Fr. 10-13-1 to Fr. 10-13-4). Compounds **1** (2.5 mg), **2** (2.6 mg) and **3** (74.2 mg) were isolated from Fr. 10-13-1 by repeated semi-preparative HPLC (YMC pack ODS-A C₁₈, 10 × 250 mm, AcCN: H₂O = 38: 62, 2 mL/min, R_t: 9.46, 26.28 and 27.40 min, respectively).

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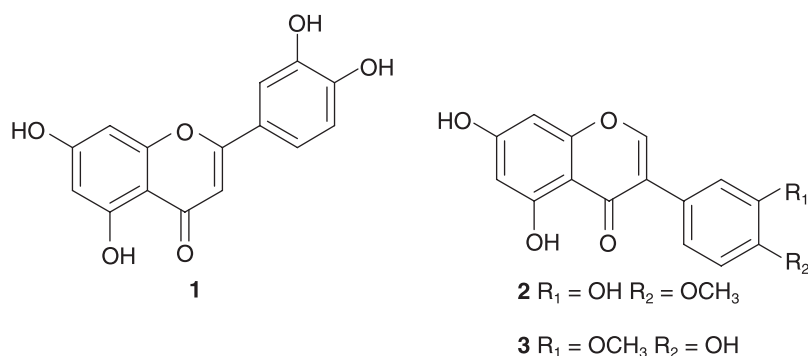


Figure 1. Structure of the compounds 1–3 isolated from *E. prostrata*. 1, diosmetin; 2, 3'-hydroxybiochanin A; 3, 3'-O-methylrobol.

Assessment of osteoblast differentiation and proliferation. ICR mice (kept at 20–23 °C; 12 h light cycle; food, Agribrand Purinar Korea, and water *ad libitum*) were provided by the Laboratory Animal Center, Seoul National University. All experiments were conducted according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Seoul National University. Murine calvarial osteoblasts were obtained from the calvariae of neonatal mice 1–2 days after birth by the sequential collagenase digestion method (Lee *et al.*, 2006). Osteoblasts were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin for 1 week and then seeded at a concentration of 1×10^4 cells/cm². Osteoblast differentiation was induced by changing the medium containing 50 µg/mL ascorbic acid and the cells were treated with vehicle or compounds to be tested. After 3 days, the media were changed with fresh medium containing each compound and maintained for a further 4 days. For the assessment of the ALP activity, the cells were rinsed with phosphate-buffered saline and lysed in 0.01% sodium dodecyl sulfate in PBS followed by sonication. After clarification by centrifugation, the cell lysates were assayed for ALP activity using an alkaline phosphatase assay kit (Youngdong Pharmaceutical Co., Korea). Each value was normalized with the protein content of cell lysate, measured using bicinchoninic acid with bovine serum albumin as a standard. The proliferation of osteoblasts was assessed by the MTT assay. The evaluation of statistical significance was determined by the one-way ANOVA test with a value of $p < 0.05$ considered to be statistically significant.

RESULTS AND DISCUSSION

In a continuation of the search for natural products having stimulatory activity on osteoblast differentiation, the methanol extract of aerial parts of *E. prostrata* was found to increase the ALP activity significantly in primary cultures of mouse calvarial osteoblasts. Bioactivity-guided fractionation of the methanol extract of *E. prostrata* resulted in the isolation of one flavonoid (1) and two isoflavonoids (2 and 3). The structures of these compounds were identified as diosmetin (1), 3'-hydroxybiochanin A (2) and 3'-O-methylrobol (3) by direct comparison of their physicochemical and spectroscopic data with those previously reported (Roberts *et al.*, 2004; Shen *et al.*, 1993; Hosny and

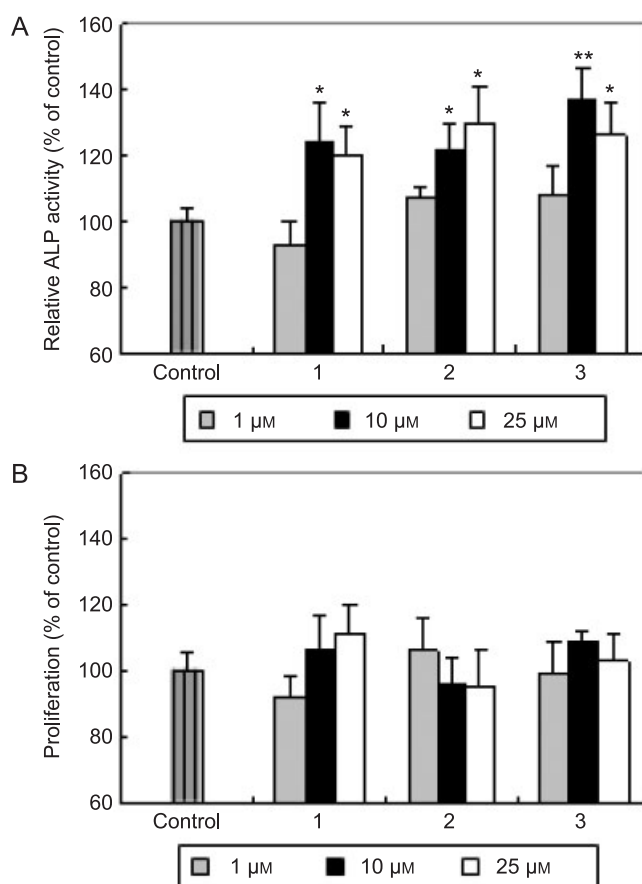


Figure 2. The stimulatory activity of compounds 1–3 isolated from *E. prostrata* on the differentiation (A) and on the proliferation (B) of primary cultures of mouse osteoblasts. Cultures were treated with each compound in the presence of 50 µg/mL ascorbic acid for 7 days. The differentiation and proliferation of mouse osteoblasts were measured by the ALP activity assay and the MTT assay, respectively, as described in Materials and Methods. (A) The ALP activity of the control was 20.1 ± 0.3 U/mg protein. The ALP activity (%) was calculated as $100 \times (\text{ALP activity of compound-treated}/\text{ALP activity of control})$. (B) The optical density (OD) of control was 0.37 ± 0.02 . Proliferation (%) was calculated as $100 \times (\text{OD of compound-treated}/\text{OD of control})$. The values are expressed as mean \pm SD of triplicate experiments. The mean value was significantly different (* $p < 0.05$, ** $p < 0.01$) from the control.

Rosazza, 1999). All three compounds are reported from this plant for the first time.

The effects of these flavonoids on osteoblast differentiation was evaluated in primary cultures of mouse calvarial osteoblasts. All three compounds significantly

increased the ALP activity at concentrations ranging from 1.0 to 25.0 μM (Fig. 2A). However, these compounds did not show significant effects on osteoblast proliferation (Fig. 2B).

Isoflavonoids are known to exert many biological and pharmacological activities. Related to bone, isoflavonoids such as daidzein and genistein have been reported to increase osteoblast differentiation, i.e. ALP activity in an *in vitro* system (Kanno *et al.*, 2004). Isoflavonoids also have exerted beneficial effects against bone loss in various experimental models (Arjmandi *et al.*, 1996) and in a clinical study (Morabito *et al.*, 2002). Consistent with the previous reports, in the present study,

two isoflavonoids and one flavonoid isolated from *E. prostrata* also exerted stimulatory activity on osteoblast differentiation. These results suggest a possibility of therapeutic potentials for osteoporosis, although the mechanism of these compounds has not been verified fully yet.

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