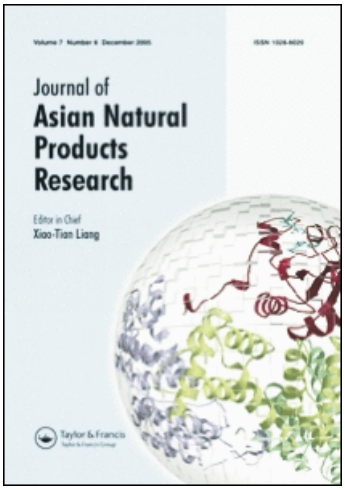

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Chrysoeriol isolated from the leaves of *Eurya ciliata* stimulates proliferation and differentiation of osteoblastic MC3T3-E1 cells

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Chrysoeriol (**1**) was isolated as the main constituent from the methanol extract of the dried leaves of *Eurya ciliata* Merr. To investigate the bioactivities of **1** on bone metabolism, its effects on the function of osteoblastic MC3T3-E1 cells were studied. Compound **1** significantly increased the growth of MC3T3-E1 cells and caused a significant elevation of alkaline phosphatase activity, collagen content, and nodule mineralization in the cells ($P < 0.05$). Our data indicate that the enhancement of osteoblast function by **1** may be useful in the prevention of osteoporosis.

Keywords: anti-osteoporosis; chrysoeriol; *Eurya ciliata*; MC3T3-E1 cell

1. Introduction

Osteoporosis remains a major public health problem, especially for the elderly. Osteoporosis is a systemic disorder characterized by low bone mass and microarchitectural deterioration of bone tissue, which leads to an increased risk of fracture [1,2]. In humans, bone is formed and resorbed in a tightly coupled process called remodeling. The bone remodeling cycle begins with the activation of resting osteoblasts on the surface of bone and marrow stromal cells. This is followed by a cascade of signals originating from the older osteoblasts to osteoclasts and their precursors [3]. After osteoclast-induced bone resorption, matrix components such as tumor growth factor-beta and insulin-like growth factor-1, as well as collagen, osteocalcin, and other protein and mineral components, are released into the microenvironment. Growth factors released by resorption contribute to the recruitment of new osteoblasts to the bone surface, which begin the process of collagen synthesis and biomineralization. After mineralization, the complete bone becomes hard and rigid with necessary mechanical properties to withstand external forces, support the body, and protect the internal organs [4]. When the remodeling process is uncoupled, a process where resorption exceeds formation, bone is lost and osteoporosis occurs. On the other hand, when formation exceeds resorption, the result is a net gain of bone.

There are several key components of the remodeling cycle that are susceptible to systemic and local alterations and which, when perturbed, can lead to deleterious changes in bone mass. In particular, activation of remodeling via osteoblasts

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and recruitment of osteoclasts represent the two most vulnerable sites in the cycle. A third site that could be altered by disease states is the osteocyte, an entombed fully differentiated osteoblast that connects to the surface osteoblasts, and likely senses mechanical stimulation. Therefore, a method that investigates the osteocyte would lead to the discovery of molecular targets, and the molecules that stimulate bone formation while inhibiting bone resorption would be more suitable for the treatment of osteoporotic diseases.

In our screening of bioactive components from Vietnamese plants, a methanol extract from the leaves of *Eurya ciliata* Merr. (Vietnamese name 'Linh lông', Theaceae) was found to exhibit considerable monoamine oxygenase inhibitory activity [5]. More recently, it has been demonstrated that this methanol extract can significantly stimulate the growth of MC3T3-E1 cells at a concentration of 3 and 0.3 μg/ml ($P < 0.05$) [6]. Phytochemical studies of the bioactive fractions led to the isolation of three flavonoids (chrysoeriol, apigenin, and quercitrin) along with myoinositol [7]. Chrysoeriol (Figure 1) was found to be the main constituent of the leaves of *E. ciliata* and its ^{13}C NMR spectral data were reassigned by 2D NMR methods including heteronuclear single quantum coherence, heteronuclear multiple bond correlation, and nuclear Overhauser enhancement spectroscopy [8]. It was also found in the leaves of some other *Eurya* species such as *E. japonica*, *E. emarginata* [9], and several plants belong to Asteraceae family including *Artemisia vulgaris* [10] and *Artemisia argyi* [11]. It displays potent antioxidant activity in various assays: lipid peroxidation, reaction with superoxide radicals, and free radical scavenging activity with 2,2-diphenyl-1-picrylhydrazyl [12]. It was also reported to inhibit the mutagenicity caused by 3-amino-1-methyl-5-*H*-pyrido[4,3-*b*]indole (Trp-P-2) in *Salmonella typhimurium* TA98 [11]. Recently, it has been found to have a selective bronchodilator effect [13] and an inhibitory effect on lipopolysaccharide-induced nitric oxide production in BV2 microglia [14]. In the present study, the *in vitro* effect of **1** on the function of osteoblastic MC3T3-E1 cells was investigated to determine its possible bioactivities on bone metabolism and prevention of osteoporosis disease.

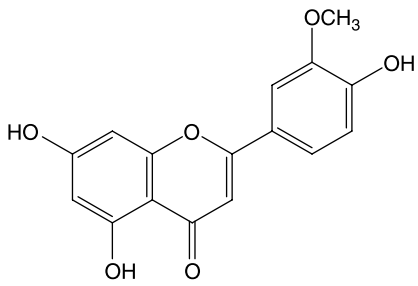

Figure 1. Structure of chrysoeriol (**1**).

2. Results and discussion

Osteoporosis associated with estrogen deficiency is the most common cause of age-related bone loss. Hormone replacement therapy (HRT) can resolve most postmenopausal problems. However, compliance with HRT is poor because of its associated risks of breast and endometrial cancers with long-term use [15]. In the search for an alternative treatment, the potential health benefits of phytoestrogens have been suggested [16]. There is considerable evidence indicating that phytoestrogens, like certain selective estrogen receptor modulators, have estrogenic action on bone and the cardiovascular system, and have antiestrogenic action on the breast and uterus [17]. Phytoestrogens have been proposed to prevent bone resorption and promote bone density [18]. Recently, chrysoeriol (**1**), a constituent of Rooibos tea, has been shown to inhibit 17β-hydroxysteroid dehydrogenase type 1, an important target for the development of drugs for the treatment of estrogen-dependent diseases [19]. In the

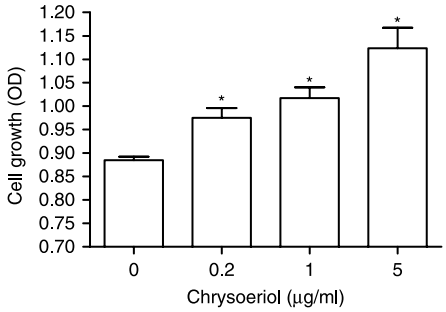

Figure 2. Effects of **1** on the viability of MC3T3-E1 cells. Data are expressed as a percentage of the control. $^{*}P < 0.05$ versus control.

present study, we investigated the effect of **1** on the function of osteoblasts using the pre-osteoblastic target cell line, MC3T3-E1, which has been well characterized as an *in vitro* model for osteoblast differentiation [20]. MC3T3-E1 cell growth was elevated significantly by the presence of chrysoeriol (0.2–5 μg/ml; Figure 2). The effect of **1** on osteoblast differentiation was first assessed by measuring alkaline phosphatase (ALP) activity, one of the major osteoblast differentiation markers. Compound **1** significantly increased the ALP activity. At 5 μg/ml, **1** increased the ALP activity up to 122% compared with that of the control (Figure 3). We further investigated the effect of **1** on collagen synthesis using Sirius red-based colorimetric assay.

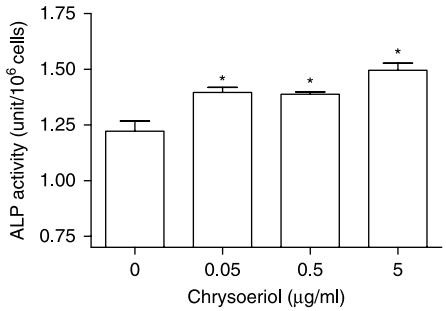

Figure 3. Effects of **1** on the alkaline phosphatase activity of MC3T3-E1 cells. Data are expressed as a percentage of the control. $^{*}P < 0.05$ versus control.

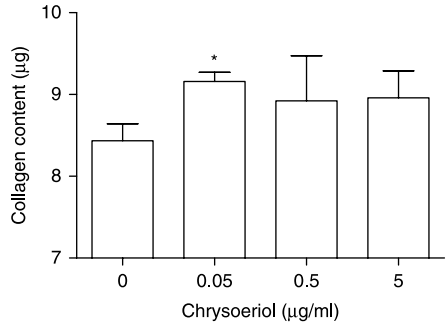

Figure 4. Effects of **1** on the collagen content of MC3T3-E1 cells. Data are expressed as a percentage of the control. $^{*}P < 0.05$ versus control.

Compound **1** significantly increased collagen synthesis at a concentration of 0.05 μg/ml (Figure 4). Next, we examined the effects of **1** on mineralization, another important process in differentiation, by measuring the calcium deposition by Alizarin red staining. Chrysoeriol showed a significant stimulatory effect on mineralization at a concentration of 0.05 μg/ml (Figure 5). At 5 μg/ml, **1** did not show any cytotoxicity. Our results demonstrate that **1** significantly increases the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

Osteoblasts are the bone-forming cells of the skeleton; they synthesize and regulate the deposition and mineralization of the extracellular matrix of bone. MC3T3-E1 cells, an osteoblast-like cell

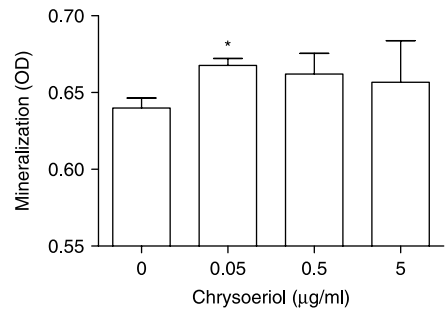

Figure 5. Effects of **1** on the mineralization of MC3T3-E1 cells. Data are expressed as a percentage of the control. $^{*}P < 0.05$ versus control.

line, have been reported to retain their capacity to differentiate into osteoblasts, and may provide very useful information about the effects of phytochemicals on the differentiation of osteoblasts [21]. ALP is one of the osteoblast phenotype markers, and has been reported to be an essential enzyme for mineralization [22]. ALP is released into the osteoid to initiate the deposit of minerals. After mineralization, the complete bone becomes hard and rigid with necessary mechanical properties to withstand external forces, support the body, and protect the internal organs [23]. The present study has demonstrated that **1** can increase ALP activity and calcium deposition in osteoblastic MC3T3-E1 cells, indicating their anabolic effect. Therefore, **1** isolated from the leaves of *E. ciliata* Merr. has direct stimulatory effects on bone formation in cultured MC3T3-E1 osteoblast cells.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a HMK apparatus (HMK; Franz Kustner, Dresden, Germany). IR spectra (KBr disk) were recorded on a FTIR spectrophotometer Nicolet Model Impact 410 (Nicolet, Madison, WI, USA) in the range of 400–4000 cm^{-1}. EI-MS spectra were measured on a 5989B MS spectrometer (Hewlett Packard, Avondale, PA, USA). NMR spectra were obtained using a Bruker AVANCE 500 spectrometer (Bruker, Karlsruhe, Germany). Chemical shifts are given in δ (ppm) with TMS as an internal standard.

3.2 Plant material

Leaves of *E. ciliata* were collected at Tam Dao mountain, Vinh Phuc Province and were identified by an experienced botanist at the National Institute of Medicinal Materials, Ministry of Health, Hanoi, Vietnam. A voucher specimen (No. VN802) has been deposited at the Department of Herbarium, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

3.3 Extraction and isolation

Dried leaves of *E. ciliata* (1.4 kg) were extracted with methanol (MeOH) at room temperature three times for 8 h each time. After removal of the solvent under reduced pressure, the crude extract (60 g) was dissolved in 1 L of H_2O to form a suspension, which was successively partitioned with *n*-hexane, chloroform, and *n*-butanol to give *n*-hexane (20 g), chloroform (6 g), and *n*-butanol residues, respectively. The *n*-butanol extract (15 g) was then subjected to flash silica gel (80–120 mesh) column chromatography using a gradient of ethyl acetate (EtOAc) and MeOH (10:1–0:1, v/v) as the eluent to give five fractions (1a–e). Fraction 1b, which was apparent as a precipitate, was filtered through a Buchner funnel and washed several times with EtOAc to produce 1400 mg (1%) of **1** as a yellow powder [7]. The purity of the compound was more than 98% by high-performance liquid chromatography analysis.

*Chrysoeriol (**1**)*

A yellow powder; mp 298–299°C; IR (KBr) ν_{max} (cm^{-1}): 3350, 3085, 1651, 1625, 1563, 1509, 1171; ^{1}H NMR (DMSO-d_6, 500 MHz) δ_H: 12.96 (s, OH-5), 10.80 (brs, OH-7), 9.95 (brs, OH-4′), 7.57 (m, H-6′), 7.56 (m, H-2′), 6.94 (d, $J = 9.0$ Hz, H-5′), 6.90 (s, H-3), 6.51 (d, $J = 2.0$ Hz, H-8), 6.20 (d, $J = 2.0$ Hz, H-6), 3.89 (s, MeO); ^{13}C NMR (DMSO-d_6, 125 MHz) δ_C: 181.8 (C-4), 164.1 (C-7), 163.6 (C-2), 161.4 (C-5), 157.3 (C-9), 150.7 (C-4′), 148.0 (C-3′), 121.5 (C-1′), 120.3 (C-6′), 115.7 (C-5′), 110.2 (C-2′), 103.7 (C-10), 103.2 (C-3), 98.8 (C-6), 94.0 (C-8), 55.9 (MeO); EI-MS: *m/z* (%) 300 (100).

3.4 Cell culture

Murine osteoblastic MC3T3-E1 cells were cultured at 37°C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. When the cells reached confluence, they were subcultured using a 0.02% EDTA–0.05% trypsin solution.

3.5 Cell viability

Cells were suspended in medium supplemented with 10% FBS and a portion of the suspension determined to contain 5×10^3 cells was added to individual wells of 48-well microplates. The plates were incubated at 37°C in a CO_2 incubator for 48 h. After discarding the culture medium and washing the cells with phosphate-buffered saline (PBS), serum-free medium containing 0.3% bovine serum albumin (BSA) and compounds at appropriate concentrations were added to the cell culture and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 48 h. Surviving cells were counted by a standard method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). A 20 μl volume of MTT in 7.2 mM phosphate buffer solution, pH 6.5 (5 mg/ml), was added to each well and the plates were incubated for an additional 2 h. After the removal of solutions in the well, dimethyl sulfoxide was added to dissolve formazan products, and the plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

3.6 Alkaline phosphatase activity

The cells were treated at 90% confluence with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to initiate differentiation. The medium was changed every 2–3 days. After 8 days, the cells were cultured with medium containing 0.3% BSA and compounds individually for 2 days. On harvesting, the medium was removed and the cell monolayer was gently washed twice with PBS. The cell number was determined microscopically and was calculated as the number of living cells (i.e. those not stained with trypan blue). The cells were lysed with 0.2% Triton X-100 and the lysate was centrifuged at 14,000 *g* for 5 min. The clear supernatant was used to measure the ALP activity, which was determined using an ALP activity assay kit (Waco, Osaka, Japan).

3.7 Collagen content

Cells were treated at 90% confluence with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The medium was changed every 2–3 days. After 8 days, the cells were cultured with medium containing 0.3% BSA and compounds for 2 days. On harvesting, the medium was removed and the cell monolayer was gently washed twice with PBS. Collagen content was quantified by Sirius red-based colorimetric assay. Cultured osteoblasts were washed with PBS, followed by fixation with Bouin's fluid for 1 h. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air dried and stained by Sirius red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm.

3.8 Calcium deposition assay

The cells were treated at 90% confluence with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. After 12 days, the cells were cultured

with medium containing 0.3% BSA and compounds individually for 2 days. On harvesting, the cells were fixed with 70% ethanol for 1 h, and then stained with 40 mM Alizarin red staining for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 10% cetylpyridinum chloride by shaking for 15 min. The absorbance of the solubilized stain was measured at 561 nm.

3.9 Statistics

The results are expressed as the mean ± SEM ($n = 4$). Statistical analysis was performed using a one-way ANOVA ($P < 0.05$) with the SAS statistical software.

4. Conclusion

The effect of **1** on bone cell development was investigated for the first time. Our data indicate that **1** stimulates MC3T3-E1 cell growth and differentiation *in vitro*. Compound **1** significantly elevates ALP activity, collagen content, and nodule mineralization in MC3T3-E1 cells ($P < 0.05$). The enhancement of osteoblast function by **1** may be useful in the prevention for osteoporosis. It is worth examining whether **1** or total flavonoids obtained from *E. ciliata* could be used as a dietary phytoestrogen in the treatment of osteoporosis and menopausal symptoms.

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