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SHORT COMMUNICATION

Effects of *Eucommia ulmoides* Extract on Longitudinal Bone Growth Rate in Adolescent Female Rats

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Eucommia ulmoides **is one of the popular tonic herbs for the treatment of low back pain and bone fracture and is used in Korean medicine to reinforce muscles and bones. This study was performed to investigate the effects of *E. ulmoides* extract on longitudinal bone growth rate, growth plate height, and the expressions of bone morphogenetic protein 2 (BMP-2) and insulin-like growth factor 1 (IGF-1) in adolescent female rats. In two groups, we administered a twice-daily dosage of *E. ulmoides* extract (at 30 and 100 mg/kg, respectively) *per os* over 4 days, and in a control group, we administered vehicle only under the same conditions. Longitudinal bone growth rate in newly synthesized bone was observed using tetracycline labeling. Chondrocyte proliferation in the growth plate was observed using cresyl violet dye. In addition, we analyzed the expressions of BMP-2 and IGF-1 using immunohistochemistry. *Eucommia ulmoides* extract significantly increased longitudinal bone growth rate and growth plate height in adolescent female rats. In the immunohistochemical study, *E. ulmoides* markedly increased BMP-2 and IGF-1 expressions in the proliferative and hypertrophic zones. In conclusion, *E. ulmoides* increased longitudinal bone growth rate by promoting chondrogenesis in the growth plate and the levels of BMP-2 and IGF-1. *Eucommia ulmoides* could be helpful for increasing bone growth in children who have growth retardation.**

Keywords: Eucommia ulmoides; herbal extract; longitudinal bone growth rate; BMP-2; IGF-1.

INTRODUCTION

Short stature is defined as having a height less than two standard deviations below the mean of the age-matched and sex-matched population (Kamboj, 2005). Short stature is partly caused by diseases such as growth hormone (GH) deficiency, Turner syndrome, Noonan syndrome, chronic renal failure, and Prader–Willi syndrome, and may occur in children born small for their gestation age. Sometimes the condition has no diagnostic explanation (idiopathic short stature) after ordinary growth evaluation.

The current methods for increasing final height include treatment with a GH, alone or in combination with an analog of gonadotropin-releasing hormone (Leschek *et al*., 2004). However, it is generally too expensive to pay for treatment of short stature. The height gain in children treated with 5 years of long-term GH was just 5.5 cm (Deodati *et al*., 2011). Furthermore, the average cost of GH therapy for 1-cm growth is about $27,000 (Meng *et al*., 2011). Treatment of children with a GH also has harmful side effects, such as prepubertal gynecomastia, arthralgia, edema, benign intracranial hypertension, insulin resistance, progression of scoliosis, slipped capital femoral epiphysis, and leukemia (Souza and Collett-Solberg, 2011).

In Korean medicine, growth disorders of children are classified largely into two categories, one being the five forms of growth delay and the other being the five forms of weakness. Treatments prescribed by Korean medicine include drugs, tonifying the liver and kidney, and strengthening of muscles and bones. *Eucommia ulmoides* is one of the most popular tonic herbs in this category of Korean medicine and is used to treat low back pain and bone fracture and to reinforce muscles and bones.

A pharmacological study of *E. ulmoides* focused on its function in promoting osteogenesis. *Eucommia ulmoides* increased osteoblastic differentiation and prevented bone loss in an ovariectomized rat model (Zhang *et al*., 2009). Geniposidic acid, geniposide, and aucubin from *E. ulmoides* have been shown to increase proliferation of osteoblasts and inhibit proliferation of osteoclasts *in vitro* (Ha *et al*., 2003). Ethanol extract of *E. ulmoides* leaves has been reported to promote the growth of MC3T3-E1 cells, which are preosteoblastic cells, and to suppress H_2O_2-induced apoptosis in rat MC3T3-E1 cells (Lin *et al*., 2011).

Endochondral ossification is the mechanism by which the long bones increase in length. At each growth plate,

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chondrocytes proliferate, undergo hypertrophy, mineralize the extracellular matrix, and finally die by apoptosis (Karsenty, 1998). They are replaced by osteoblasts brought in by metaphyseal blood vessels, which begin the deposition of bone tissue (Karsenty, 1998). Because components in *E. ulmoides* extract activate osteoblast differentiation, we hypothesized that treatment with *E. ulmoides* extract would increase longitudinal bone growth rate.

Longitudinal bone growth is the result of chondrocyte proliferation in the growth plates and their conversion into new bone (Guyton, 2000). If chondrocyte proliferation is inhibited, bone growth is unstable and produces abnormal bone lengths. During chondrocyte proliferation and hypertrophy, bone morphogenetic protein 2 (BMP-2) and insulin-like growth factor 1 (IGF-1) increase chondrocyte proliferation and hypertrophy in the growth plate (Mackie *et al.*, 2011). It is unclear whether *E. ulmoides* extract increases chondrocyte proliferation and hypertrophy and whether the expressions of BMP-2 and IGF-1 are affected.

In this study, we examined whether treatment of adolescent female rats with *E. ulmoides* extract increases longitudinal bone growth rate and induced the expressions of BMP-2 and IGF-1.

MATERIALS AND METHODS

Plant material. *E. ulmoides Oliv.* cortex was purchased from Yaksudang Co (Seoul, Korea). It was identified by Dr Hocheol Kim, Department of Herbal Pharmacology, College of Korean Medicine, Kyung Hee University where the voucher specimen (#HP009) is deposited.

Preparation of samples. *E. ulmoides* (50 g) was extracted with 70% ethanol (500 mL) for 3 h at 80 °C in a reflux apparatus. The extracts were filtrated and concentrated under reduced pressure, and samples were lyophilized to yield a dark brown powder. The yield of extracts was 13.9%. The quantitative authentication of *E. ulmoides* was performed by a high performance liquid chromatography (HPLC) analysis system equipped with a Waters 1525 pump, a 2707 autosampler, and a 2998 PDA detector. The chromatic separation was achieved at 40 °C on Waters Sunfire™ C_{18} (250 mm × 4 mm i.d., 5 μm particle size). The detection was carried out by UV wave length that was set at 236 nm. The run time was set at 60 min, the flow rate was 1.0 mL/min, and the sample injection volume was 10 μL. Mobile phases A and B were 0.5% H_3PO_4 (v/v) and CH_3CN, respectively. Isocreatic elution was as follows: 0–60 min 8% B. In HPLC analysis, compound was identified in *E. ulmoides*: geniposide. The content of geniposide was calculated for standardization. *Eucommia ulmoides* was standardized to contain 8.9 mg/g geniposide. An HPLC chromatogram and the structures of the constituent compounds are shown in Fig. 1.

Animals. Four-week-old female Sprague–Dawley rats, weighing 70 ± 10 g each, were used (Samtako Co, Osan, Korea). The experimental procedures were performed in accordance with the animal care guidelines of the Kyung Hee University's Institutional Animal Care and Use Committee (KHUASP (SE)-10-034). Animals were housed under controlled temperature (23 ± 2 °C), relative humidity (55 ± 10%), and lighting (07:00–19:00 h) conditions, with food and water made available *ad libitum*.

Treatment. After 1 week of acclimatization, rats were divided into three groups: control, *E. ulmoides* 30 mg/kg, and *E. ulmoides* 100 mg/kg. Vehicle or *E. ulmoides* was administered orally twice daily for four consecutive days. On the fifth day, all rats were killed under ether anesthesia for tissue analysis.

Measurement of longitudinal bone growth rate. To investigate the effect on longitudinal bone growth rate, tetracycline was used as a fluorescence marker to label the bone line on the surface of the tibia. Tetracycline plays the role of fluorescent dye under ultraviolet illumination. Tetracycline hydrochloride (20 mg/kg, Sigma Chemical Co, St. Louis, USA) was injected intraperitoneally to rats 48 h before sacrifice. The dissected tibias were fixed in 4% paraformaldehyde for 48 h and undergone decalcification by immersion in 10% ethylene diamine tetra acetic acid solution for 24 h (Sigma Chemical Co, St. Louis, USA). After dehydration by immersion in 30% sucrose for 1 day, each bone sample was sectioned longitudinally at a thickness of 40 μm with a sliding microtome (HM440E, Zeiss, Walldorf, Germany). The amount of increase in longitudinal bone growth was assessed by measuring the length between the fluorescent lines formed by tetracycline injected 48 h before sacrifice

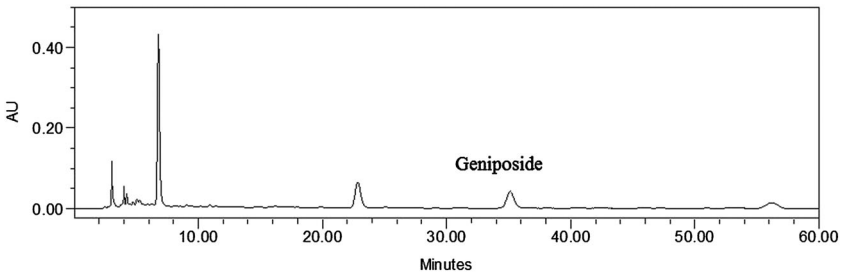

Figure 1. HPLC chromatogram for standardization of *E. ulmoides*. Detection was performed by using a photodiode array detector. *X*-axis is retention time; *Y*-axis is wavelength, and *Z*-axis is absorbance unit. Analytical conditions were as follows: column, Sunfire™ C_{18}; mobile phase, solvent A (0.5% H_3PO_4) and solvent B (CH_3CN); flow rate, 1.0 mL/min; program, 0–60 min 8% B.

and the epiphyseal end line of the growth plate. Length between two lines was measured by using Image J software (NIH, Maryland, USA). Focus was placed between the epiphyseal plate and the fluorescent band formed by chelating of tetracycline and calcium on the epiphyseal plate corresponding to injection of tetracycline, which was visible by using a fluorescence microscope (Olympus, Tokyo, Japan). The mean values were from three different sections within the fluorescent band gap.

Measurement of growth plate height. Cresyl violet was used to stain the chondrocytes in the growth plate of samples. Each sample was sectioned longitudinally at a thickness of 40 μm with a sliding microtome as described previously, and they were made slides; 0.5% cresyl violet solution used for the sections was made as follows. Cresyl violet was obtained from Sigma (Sigma Chemical Co, St. Louis, USA). Cresyl violet 2.5 mg distilled water 300 mL, 1 M sodium acetate (30 mL of 13.6 g/92 mL, and 1 M acetic acid (170 mL of 28.95 mL glacial acetic acid/500 mL distilled water) were mixed for 7 days on magnetic below. The slides were dipped into distilled water for 3 min, cresyl violet for 5 min, 50% ethanol for 3 min, 75% ethanol for 3 min, 90% ethanol for 3 min, 100% ethanol for 3 min, and xylene for 10 min mounted with permount solution and cover glass. The growth plate height was measured in three different areas per sample by using Image J software, for each of the three sections, and the average value was calculated. The cell of resting, proliferative, and hypertrophic zone was observed.

Measurement of BMP-2 and IGF-1 in growth plates. Tissue sections were washed twice in 0.1 M phosphate buffer saline (PBS) and washed twice in 1% triton X-100 (Sigma Chemical Co, St. Louis, USA) for 15 min then washed twice with 0.5% of bovine serum albumin (BSA) dissolved in PBS (BSA, Sigma Chemical Co, St. Louis, USA) for 15 min. The sections were then incubated with goat BMP-2 primary antibody and rabbit IGF-1 primary antibody (1:200, Santa Cruz Biotechnology, CA) overnight at room temperature in a humid chamber. After 24 h, sections were then washed two times with 0.5% BSA in PBS and then incubated with the biotinylated anti-goat secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and biotinylated anti-rabbit secondary antibody (1:200, Jackson Immuno Research Laboratories, USA) for 1 h, respectively. After being washed twice with PBS for 15 min, the sections were incubated with avidin-biotinperoxidase complex (1:100, Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After another washing with PBS, the sections were stained and reacted with 0.05% 3, 3-diaminobenzidine solution containing hydrogen peroxide in PBS. The reaction was stopped by washing with PBS; the slides were then dehydrated with the use of 50%, 75%, 95%, and 100% ethanol and xylene in order. The sections were then mounted on glass slides with permount medium solution (Fisher Scientific, Springfield, NJ, USA). Micrographs of sections were taken.

We adapted a method described by Reinecke *et al.* (2000) for quantifying immunohistochemistry staining, with slight modifications. Growth plate areas were photographed by microscope (Olympus, Tokyo, Japan). On these photographs, the total number of chondrocytes in two parallel columns was counted, and the percentage of labeled cells was determined. This procedure was performed twice to obtain a mean value for each of the two columns in each tibial section. Measurements and calculations of quantitative evaluation were obtained by using Image J software.

Statistical analysis. All the data were presented as mean ± SD. The effects of the different treatments were compared by Student's *t*-test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

RESULTS

Effect of *E. ulmoides* on longitudinal bone growth rate

The effect of *E. ulmoides* on longitudinal bone growth rate was assessed by taking measurements of the gap between the growth plate and the band formed by tetracycline at three different locations to obtain an average number (Fig. 2 (1)). At the dose of 100 mg/kg, *E. ulmoides* caused a significant acceleration of longitudinal bone growth rate, which was 373.1 ± 24.4 μm/day (6.4%, $p < 0.01$) compared with the control group, which was 350.8 ± 18.5 μm/day (Fig. 2 (1)). At the dose of 30 mg/kg, *E. ulmoides* caused an acceleration of longitudinal bone growth rate of 360.5 ± 23.5 μm/day (2.8%) compared with the control group. These data imply that *E. ulmoides* showed a dose-dependent and significant increase in the longitudinal bone growth rate compared with the control.

Effect of *E. ulmoides* on growth plate height

Longitudinal bone growth rate depends on a complex synchronization of the rate of proliferation, advancement and development of hypertrophy, which results in the longitudinal expansion and progression of the growth plate. The heights of the total growth plates were measured at three different locations within the growth plate for each sample and animal in each group by histological study. The overall growth plate zone was divided to the resting zone (RZ, a layer of small, round cells irregularly arranged), the proliferative zone (PZ, wherein the cells divide along the long axis of the bone in regular columns), and the hypertrophic zone (HZ, large, glycogen-filled cells). The proximal tibia growth plate in the control group was 491.8 ± 29.9 μm; the *E. ulmoides* group (100 mg/kg, p.o.) was 508.9 ± 42.7 μm (3.4%; Fig. 2 (2)). The difference between the control and *E. ulmoides* group for the growth plate height was statistically significant. The size of the cells in the proliferation zone and hypertrophic zone was observed as somewhat larger than the control group (Fig. 2 (2)). This result may be related with the proliferation of cartilage cells.

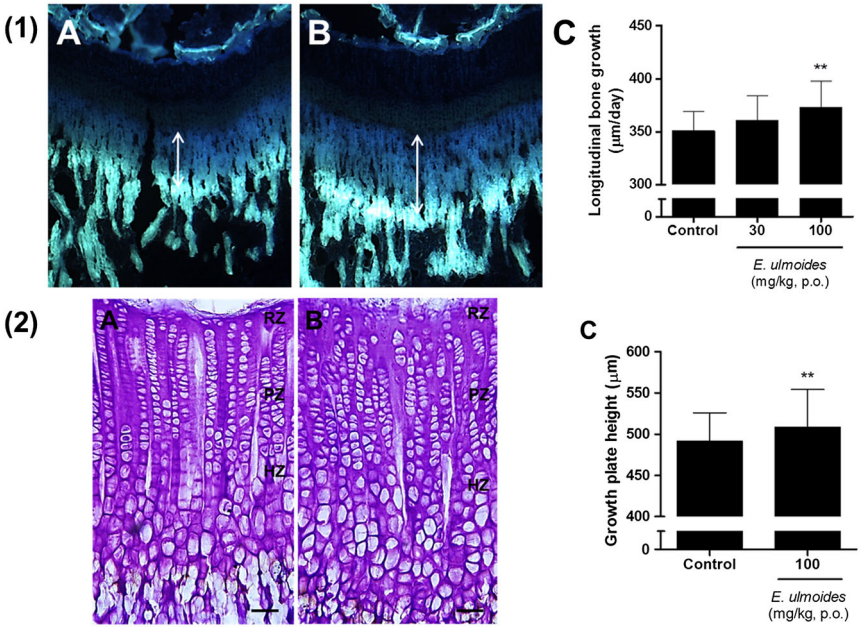

Figure 2. (1) Effect of *E. ulmoides* on longitudinal bone growth in adolescent female rats (A and B). Fluorescence photomicrographs of longitudinal sections at the growth plate in the proximal tibia. The fluorescent line corresponds to the injection of tetracycline (20 mg/kg), which binds with calcium and can be detected by ultraviolet illumination. The arrow between the fluorescent line formed by tetracycline and the epiphyseal end line of the growth plate indicates the length of bone growth during 48 h. Control group (A) and *E. ulmoides* (100 mg/kg) group (B). (C) Effect of *E. ulmoides* (100 mg/kg) on longitudinal bone growth in adolescent female rats: control group and *E. ulmoides* (30 and 100 mg/kg) group. Each value is the mean ± SD of ten animals. $**p < 0.01$, significant differences compared with control and (2) effect of *E. ulmoides* on growth plate height in adolescent female rats. Photographs of growth plate in a proximal tibia (A and B). Sections were stained with cresyl violet. Control group (A) and *E. ulmoides* (100 mg/kg) group (B): RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone. (C) Effect of *E. ulmoides* (100 mg/kg) on growth plate height in adolescent female rats: control group and *E. ulmoides* (30 and 100 mg/kg) group. Each value is the mean ± SD of ten animals. $**p < 0.01$, significant differences compared with control. The scale bar is 50 μm. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

Effects of *E. ulmoides* on BMP-2 and IGF-1 expression

Immunohistochemical studies were performed to evaluate the expression of BMP-2 and IGF-1 in the three major principle zones of the growth plate. Bone morphogenetic protein 2 staining showed the highest change in the cytoplasm of the proliferative zone and hypertrophic zone (Fig. 3). Treatment of *E. ulmoides* increased markedly the BMP-2 expression in proliferative zone ($p < 0.05$) and hypertrophic zone ($p < 0.05$) of growth plate (Table 1). Insulin-like growth factor 1 staining showed the highest change in the cytoplasm of the resting zone, proliferative zone, and hypertrophic zone (Fig. 3). Treatment of *E. ulmoides* increased markedly the IGF-1 expression in resting zone ($p < 0.01$), proliferative zone ($p < 0.05$), and hypertrophic zone ($p < 0.05$) of growth plate (Table 1). Both the number and intensity of BMP-2 and IGF-1 positive cells were increased in the proliferative zone and hypertrophic zone of growth plate

DISCUSSION

In the present study, *E. ulmoides* extract increased longitudinal bone growth rate and growth plate height in adolescent female rats and upregulated the expressions of BMP-2 and IGF-1 in the proliferative zone and the hypertrophic zone.

Long bone growth, especially of the femur, tibia, and fibula in the leg, is usually used for evaluating growth processes (Wong and Rabie, 2006; Petrovecki *et al.*, 2007). Among the long bones, the tibia is the best indicator of stature (Duyar and Pelin, 2003). In this study, the longitudinal bone growth rate of the tibia was assessed by measuring the length between the fluorescent line formed by tetracycline and the epiphyseal end line of the growth plate. The group treated with *E. ulmoides* extract exhibited significantly greater (6.4%) longitudinal bone growth rate of the tibia compared with that exhibited by the control group. This result suggests that the *E. ulmoides* extract increases longitudinal bone growth rate.

The growth plate, which is located at the distal end of the bone, is the main location where longitudinal bone growth occurs owing to the stimulation of chondrocyte proliferation. The chondrocytes of the growth plate are constantly dividing by mitosis. Because the synchronized processes of chondrocyte proliferation and cartilage ossification lead to longitudinal bone growth (Weise *et al.*, 2001), the rate of longitudinal bone growth is regulated by the rate of chondrocyte proliferation on one side of the growth plate (Hunziker, 1994; Bass *et al.*, 1999).

The proliferation of chondrocytes in the growth plate is a major component of growth plate-mediated growth (Villemure and Stokes, 2009). The rate of chondrocyte proliferation in our experiments was determined by measuring the growth plate height. The tibial growth plate height was significantly greater by 3.4% in the *E. ulmoides*-treated group compared with that in the control group. This suggests that *E. ulmoides* extract increases chondrocyte proliferation in the growth plate.

Treatment with *E. ulmoides* extract increased the levels of local BMP-2 in the proliferative and hypertrophic zones and IGF-1 in the resting, proliferative, and

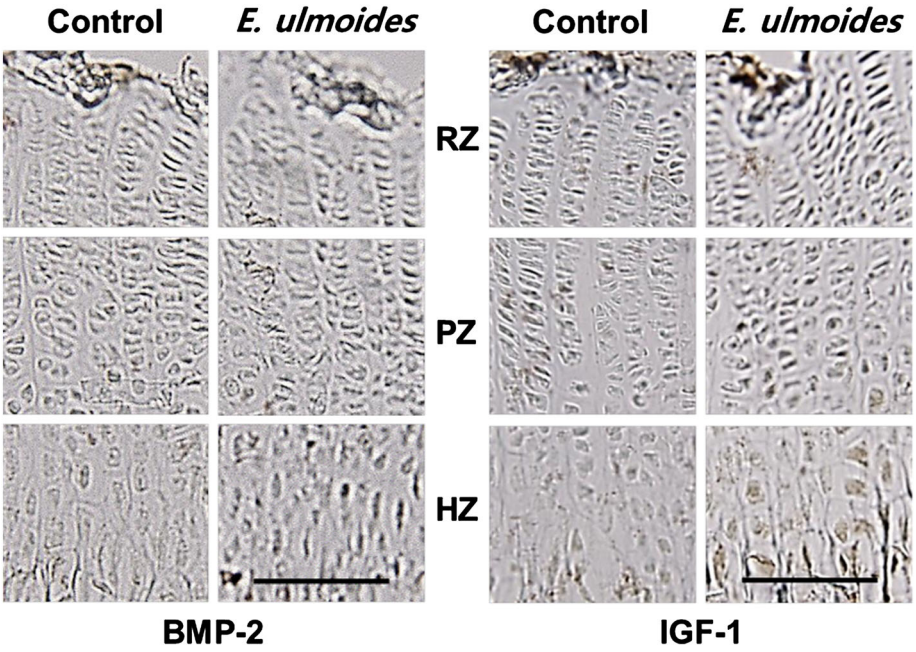

Figure 3. Immunohistochemical localization of bone morphogenetic protein 2 and insulin-like growth factor 1 on the growth plate in adolescent female rats: control, control group; *E. ulmoides*, *E. ulmoides* (100 mg/kg) group: RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone. The scale bar is 100 μm.

Table 1. Percentage of labeled chondrocytes in growth plate cartilage

	Zone	Control	*Eucommia ulmoides*
BMP-2	Resting zone	62.3 ± 6.3	77.5 ± 6.4
	Proliferative zone	67.0 ± 3.4	84.6 ± 7.1*
	Hypertrophic zone	48.1 ± 10.2	81.6 ± 5.0*
IGF-1	Resting zone	49.0 ± 1.6	66.6 ± 5.4**
	Proliferative zone	69.2 ± 1.6	79.4 ± 4.8*
	Hypertrophic zone	57.7 ± 7.1	77.6 ± 10.1*

BMP-2, bone morphogenetic protein 2; IGF-1, insulin-like growth factor 1.

Data are shown as mean ± SD values. $*p < 0.05$; $**p < 0.01$, compared with the control, by Student's *t*-test.

hypertrophic zones of the growth plate. Bone morphogenetic protein 2 expressed in the growth plates of long bones (Vortkamp *et al.*, 1998) accelerates longitudinal bone growth by stimulating growth plate chondrocyte proliferation and chondrocyte hypertrophy via the Wnt/β-catenin signaling pathway (Li *et al.*, 2011). Insulin-like growth factor 1, which is generated by the liver under the control of GHs, can regulate cell growth and development (Delafontaine *et al.*, 2004). Insulin-like growth factor 1 stimulates the proliferation and differentiation of chondrocytes (Mackie *et al.*, 2011). This suggests that treatment with *E. ulmoides* extract increases longitudinal bone growth rate by promoting chondrogenesis and stimulating chondrocyte differentiation through the upregulation of BMP-2 and IGF-1 expression.

In conclusion, the *E. ulmoides* extract increased longitudinal bone growth rate by promoting chondrocyte proliferation and stimulating chondrocyte differentiation, via the upregulation of BMP-2 and IGF-1 expressions in the growth plate. *Eucommia ulmoides* may be helpful for increasing bone growth rate in children who have growth retardation. These results lead to the assumption that *E. ulmoides* extracts have the potential to induce height increase; however, further research, including clinical trials, is necessary.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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