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# Limonin enhances osteoblastogenesis and prevents ovariectomy-induced bone loss

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ABSTRACT

Limonin, the most prevalent limonoid in citrus fruits, is widely used as a dietary supplement because of its biological activities. Although studies have shown that limonin inhibits bone resorption in osteoclasts and improves bone quality in orchidectomised rats, the anti-osteoporosis activity of limonin in ovariectomised (OVX) animal models is still unknown, and its exact molecular mechanisms remain undefined. Therefore, the effect of limonin on osteoblastogenesis in MC3T3-E1 cells and an OVX-induced osteoporosis rat model was investigated. Limonin stimulated alkaline phosphatase (ALP) activity, mineralisation, and increased osteoblast differentiation gene marker expression via extracellular signal-regulated kinase (ERK) and p38 signalling in osteoblastic MC3T3-E1 cells. In animals, limonin decreased OVX-induced body weight increase, affected the trabecular bone structure and biochemical properties, and facilitated bone mineral density and content regulation. These findings demonstrated that limonin has beneficial bone metabolism effects based on bone formation and might have potential as a functional food ingredients and a dietary supplement for osteoporosis.



## 1. Introduction

Bone is a complex, rigid organ that constantly repeats the cycle of formation and resorption by osteoblasts and osteoclasts, respectively, to facilitate growth and repair (Genant et al., 2007). Osteoporosis is the most frequent bone remodelling imbalance in which bone resorption exceeds formation, and is characterised by a reduction in bone mass, microarchitectural deterioration, and bone composition changes, which finally lead to fractures

*Abbreviations:* ARS, alizarin red stain; BMC, bone mineral content; BMD, bone mineral density; BV/TV, bone volume over tissue volume; OVX, ovariectomised; SMI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; VOI, volume of index

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*Chemical compounds:* Limonin (PubChem CID: 179651); Penicillin-streptomycin (PubChem CID: 86591708); L-ascorbic acid (PubChem CID: 54670067); β-glycerophosphate (PubChem CID: 16219387); Dimethyl sulphoxide (PubChem CID: 679); Ethylenediaminetetraacetic acid (PubChem CID: 60499); Sodium fluoride (PubChem CID: 5235); Sodium vanadate (PubChem CID: 61671).

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(Cooper, Westlake, & Harvey, 2006). Most patients with osteoporosis are postmenopausal women with associated ovarian hormonal deficiencies following a rapid hormone-related bone loss. Therefore, osteoporosis has become a serious public health problem causing significant health care issues worldwide (Renno et al., 2006). Currently, there are various treatment options available to reduce osteoporosis-induced bone loss and fracture risk. Treatment of osteoporosis targets either the preservation of skeletal mass by suppressing osteoclastic bone resorption or reversal of bone loss by stimulating osteoblastic bone formation (Putnam, Scutt, Bicknell, Priestley, & Williamson, 2007). Most osteoporosis treatment regimens largely involve the use of anti-resorptive agents such as bisphosphonates, oestrogen, and raloxifene rather than anabolic agents (bone formation) such as parathyroid hormone (PTH) (Hodsman et al., 2005; Miller, 2008). Although two types of therapeutic agents can decrease the frequency of fractures and increase bone mineral density (BMD), they have limitations such as high cost and various side effects. Therefore, there are numerous studies currently being conducted in the search for herbal products and traditional foods that promote bone mass increase and decrease postmenopausal bone fracture. Recently, natural products and compounds have gained much attention and focus as potential alternative approaches for osteoporosis treatment by stimulating osteoblast differentiation (Wang et al., 2014).

Several epidemiological studies have reported that increased consumption of fruits and vegetables, which are rich in vitamin C, flavonoids, and limonoids, has positive health-maintaining effects (Kaur & Kapoor, 2001; Liu, 2003; Qi et al., 2015; Van Duyn & Pivonka, 2000). Limonin is widely used as a dietary supplement and is the most prevalent limonoid found in citrus fruits such as oranges, lemons, grapefruit, limes, and yuza (Mahmoud, Gamal, & El-Fayoumi, 2014). Several studies have shown that limonin possesses numerous biological activities such as antioxidant, anticarcinogenic, anti-inflammatory, and antiviral (El-Readi, Hamdan, Farrag, El-Shazly, & Wink, 2010; Han et al., 2011; Langeswaran, Gowtham, Revathy, & Balasubramanian, 2012). Limonoids, including limonin, may protect against bone resorption *in vitro* (Jayaprakasha et al., 2007; Jayaprakasha & Patil, 2007; Li et al., 1998; Mandadi, Jayaprakasha, Bhat, & Patil, 2007; Yu et al., 2005). Furthermore, limonin improves the bone quality of orchidectomised rats (Mandadi et al., 2009). However, the effects of limonin on bone formation in osteoblasts and OVX-induced osteoporotic rats have not been elucidated. Therefore, we sought to characterise the effects of limonin on osteoblastogenesis activity, as well as assess its preventive and therapeutic effects in an osteoporosis rat model.

## 2. Materials and methods

### 2.1. Reagents and chemicals

Alpha minimum essential medium (αMEM), foetal bovine serum (FBS), penicillin-streptomycin (PS), and phosphate-buffered saline (PBS) were obtained from Gibco BRL (Grand Island, NY, USA). Antibodies against β-actin (sc-47778) and secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against phospho-extracellular signal-regulated kinase (p-ERK, 4377S), ERK (4695S), p-p38 (9211S), p38 (9212S), p-c-Jun

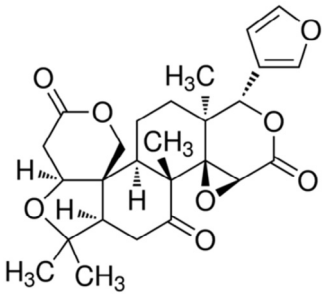

**Fig. 1 – Structure of limonin.**

N terminal kinase (p-JNK, 9251S), and JNK (9252S) were purchased from Cell Signaling Technology (Danvers, MA, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), alizarin red S (ARS, A5533), and limonin (L9647, purity > 90%, Fig. 1) were purchased from Sigma Chemical Corp., (St. Louis, MO, USA) while von Kossa (calcium stain, ab150687) was purchased from Abcam (Cambridge, UK).

### 2.2. Cell culture and osteogenic differentiation

MC3T3-E1 subclone 4 cells were cultured in αMEM containing 10% FBS and 1% PS without L-ascorbic acid at 37 °C under an atmosphere of 5% $CO_2$, and then seeded in a 24-well plate at a density of $2 \times 10^4$ cells/well. When the cell attained confluence, osteogenic differentiation was induced with differentiation medium containing 50 μg/mL L-ascorbic acid and 10 mM β-glycerophosphate with limonin, and the medium was replaced every two days.

### 2.3. Cell viability

Cell viability was measured using the MTT assay. Briefly, MC3T3-E1 cells were seeded in a 96-well plate at $1 \times 10^4$ cells in 100 μL of medium/well. After a 24-h incubation, the cells were treated with varying concentrations of limonin for 24 h, and then 20 μL of a 5 mg/mL MTT solution was added to each well, followed by further incubation at 37 °C for 2 h. The purple formazan formed in living cells was solubilised with dimethyl sulfoxide (DMSO). Cell viability was subsequently calculated by measuring the absorbance at 540 nm using a microplate reader (Infinite M200 PRO, Tecan Group Ltd., Männedorf, Switzerland).

### 2.4. ALP activity

To measure ALP activity, the cells were incubated for 6 days, washed twice with PBS twice, and lysed with 0.2% triton-X-100 lysis buffer. Then, the lysate was sonicated, agitated at 4 °C for 10 min, and then centrifuged at 2500 × *g* at 4 °C for 10 min. The supernatants were collected, and ALP activity was measured using a SensoLyte *p*NPP Alkaline Phosphatase Assay Kit (AnaSpec, Fremont, CA, USA) as directed by the manufacturer.

### 2.5. Mineralisation assay

To measure mineralisation, cells were cultured in differentiation medium with limonin for 21 days, stained with ARS or von