

Fructus Ligustri Lucidi ethanol extract inhibits osteoclastogenesis in RAW264.7 cells via the RANKL signaling pathway

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Abstract. *Fructus ligustri Lucidi* (FLL) is the fruit of *Ligustrum lucidum Ait* and a traditional Chinese medicine, primarily known for its role in osteoporosis prevention and treatment. The present study aimed to elucidate the effect and underlying mechanism of action of ethanol extract of FLL on osteoclast differentiation and bone resorption, and to identify the active compounds within it. RAW264.7 murine monocyte/macrophage cells were stimulated with the receptor activator of nuclear factor κ B ligand (RANKL) to induce osteoclast differentiation *in vitro*. The present study demonstrated that FLL extract and its two primary components, oleanolic acid (OA) and ursolic acid (UA), significantly suppressed RANKL-induced tartrate resistant acid phosphatase (TRAP) activity and multinucleate osteoclast formation without inducing cytotoxicity; however, no effect was observed on the apoptosis of mature osteoclasts. Additionally, RANKL-induced mRNA expression levels of the key transcription factors, tumor necrosis factor receptor associated factor-6, nuclear factor of activated T cell-c1 and c-Fos, and the osteoclast markers, TRAP, cathepsin K and matrix metalloproteinase-9 were suppressed by FLL, OA and UA. However, no effect was observed on RANKL-induced mRNA expression levels of Src. These results demonstrated that FLL may inhibit osteoclastogenesis in RAW264.7 cells via RANKL signaling pathways. OA and UA are active compounds in inducing this effect; however, their specific roles remain to be elucidated.

Introduction

The World Health Organisation (WHO) defines osteoporosis as a systemic skeletal disease characterized by low bone

density and micro-architectural deterioration of bone tissue, which renders bone susceptible to fractures (1). Osteoporosis has become a primary public health concern worldwide, as its incidence increases with the ageing population (2). As a dynamic system which continuously undergoes reconstruction, the bone system maintains a balance between osteoblastic bone formation and osteoclastic bone resorption (3). Increased osteoclastogenesis or osteoclastic activity may cause an imbalance in bone remodeling and accelerate bone loss (4), as demonstrated in various skeletal disorders, including osteoporosis, rheumatoid arthritis and Paget's disease of bone (5). Thus, osteoclasts are potential targets for anti-resorptive agents.

Osteoclasts are multinucleate cells derived from hematopoietic cells (6). Receptor activator of nuclear factor κ B ligand (RANKL) is important in osteoclastogenesis and activation of mature osteoclasts (7). Binding of RANKL to its receptor RANK leads to the recruitment of tumor necrosis factor receptor associated factor-6 (TRAF-6) (8), thus activating downstream signaling molecules, including mitogen-activated protein kinases (MAPK), Src and the subsequent transcription factors, nuclear factor of activated T cell-c1 (NFAT-c1) and c-Fos (9). This ultimately leads to the expression of genes involved in osteoclast differentiation and bone resorption, including tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK) and matrix metalloproteinase-9 (MMP-9) (9,10). This suggests that agents that inhibit RANKL signaling may provide therapeutic potential in the treatment of diseases where bone loss is a symptom (11,12).

In recent years, traditional Chinese medicines have become increasingly investigated in the treatment and prevention of osteoporosis (13). Numerous studies have revealed that traditional Chinese medicines have beneficial effects *in vivo*, in animal models of osteoporosis and *in vitro*, in models of osteoclast differentiation (14-16). In contrast to long-term hormone replacement therapy, which may cause estrogen-like side-effects (17), traditional Chinese medicines are prepared from natural plants and are considered to have fewer side-effects (18), thus providing patients with treatment alternatives.

Among these natural herbal medicines, *Fructus ligustri Lucidi* (FLL), the fruit of *Ligustrum lucidum Ait*, is commonly used to tonify kidney and strengthen bone (19). Certain studies have revealed the positive effects of FLL in the improvement

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of bone properties, modulation of bone turnover, improvement of calcium balance in rats (20) and promotion of osteogenesis of mesenchymal stem cells (21). Previous studies conducted by our laboratory demonstrated that an ethanol extract of FLL may increase bone mineral density and improve bone mechanical properties in growing female rats (22), and upregulate calcium absorption-associated gene expression in the kidney and duodenum (23). In addition, it has been revealed to downregulate the gene expression ratio of RANKL/osteoprotegerin in MC3T3-E1 cells (an osteoblast-like cell line) (24). However, the effect of FLL on osteoclast differentiation and bone resorption, and its underlying mechanism, remain to be elucidated, and the active compounds present in FLL have not yet been identified. Oleanolic acid (OA) and ursolic acid (UA) are two primary active components in FLL extract. Certain studies have indicated that OA (or its derivative) may inhibit osteoclastogenesis and mRNA expression levels of bone-associated genes *in vitro* (24,25); however, the effect of UA on osteoclast differentiation and bone resorption, to the best of our knowledge, remains to be elucidated.

The present study aims to elucidate the effect of FLL on osteoclasts, and its underlying mechanism, and to identify the active ingredients. RANKL-induced RAW264.7 murine monocyte/macrophage cells underwent osteoclastic differentiation *in vitro*. The inhibitory effect on osteoclastogenesis was assessed by measuring the expression levels of osteoclast-associated genes.

Materials and methods

FLL extract. FLL extract was prepared by Layn Natural Ingredients Corporation (Guilin, China). Briefly, dried and powdered crude FLL seeds were extracted twice with 70% ethanol. They were subsequently filtered and concentrated under reduced pressure and lyophilized into powder at a yield of 20% by weight of the starting materials. Two primary compounds of FLL extract, OA and UA, were detected and quantified with ultra-performance liquid chromatography (UPLC). A photo-diode array (PDA) detector with 210 nm wavelength and a BEH C18 column (2.1x100 mm; 1.7 μ m) were used. The separating conditions were as follows: Methanol, water and formic acid were selected as the mobile phase at a volume ratio of 80:20:0.1 and the flow rate was 0.4 ml/min. Finally, they were confirmed by retention time compared with standard solutions.

Cell culture. The RAW264.7 murine monocyte/macrophage cell line was obtained from the Cell Resource Center, Peking Union Medical College (Beijing, China). The cells were maintained in high glucose Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 μ g/ml; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Incubations were performed at 37°C in 5% CO₂. The medium was replaced every day and the cells were subcultured every 3 days. FLL, OA and UA were dissolved in 0.1% dimethyl sulfoxide (DMSO; Amresco, LLC, Solon, OH, USA) and further diluted with cell culture medium to an appropriate concentration. In addition, 0.1%

DMSO was added to untreated cells and control groups and demonstrated no effect.

Assesment of cytotoxicity. To evaluate the cytotoxicity of the FLL extract along with OA and UA, cell viability assays on RAW264.7 cells were conducted via conventional MTT (Amresco, LLC) assays. Briefly, RAW264.7 cells were seeded in 96-well plates (1x10³ cells per well) and cultured overnight. Following this, the medium was replaced with media containing various concentrations of FLL, OA or UA. Following a 24-h incubation period, 5 mg/ml MTT (Amresco, LLC) solution was added and the plates were incubated for a further 4 h. At the end of the incubation period, the medium was replaced with 150 μ l DMSO per well for solubilization of formazan crystals and the optical density values were measured at a wavelength of 540 nm, using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Osteoclast differentiation of RAW264.7 cells. For the differentiation of RAW264.7 cells into multinucleate osteoclasts, the cells were seeded into a 96-well plate at a density of 3x10³ cells per well and incubated overnight. Following this, 50 ng/ml RANKL (PeproTech, Inc., Rocky Hill, NJ, USA) was added into the medium, which was replaced every 3 days. After 5 days, the multinucleate cells had differentiated into mature osteoclasts.

TRAP-positive multinucleate cell staining and TRAP activity assay. In the present study, TRAP was used as a marker of osteoclasts. The RAW264.7 cells were seeded into a 96-well plate and incubated overnight. Following this, 50 ng/ml RANKL and various concentrations of FLL (10, 25, 50 and 100 μ g/ml), OA (0.36, 0.89, 1.78 and 3.56 μ g/ml) and UA (0.10, 0.26, 0.52 and 1.03 μ g/ml) were added into the medium. Concentrations of OA and UA were determined based on concentrations of FLL. Every concentration of UA or OA was consistent with its proportion in the FLL extract (demonstrated by UPLC results). After 5 days of culture, the medium in the 96-well plate was transferred to a new plate for the TRAP activity assay. The cells were washed twice with pre-warmed PBS. For the TRAP activity assay, an Acid Phosphatase assay kit (BioVision, Inc., Milpitas, CA, USA) was used. Briefly, p-nitrophenyl was used as a phosphatase substrate that becomes yellow ($\lambda_{\text{max}}=405$ nm) when dephosphorylated by acid phosphatase. The TRAP staining method involved cells fixed in 4% paraformaldehyde for 20 min, followed by staining for TRAP using a Leukocyte Acid Phosphatase kit (Sigma-Aldrich, Merck Millipore) according to the manufacturer's protocol. The TRAP-positive cells appeared dark red and those containing ≥ 3 nuclei were defined as osteoclasts. The number of osteoclasts per well was counted and imaged using a light microscope (Olympus Corporation, Tokyo, Japan).

Apoptosis assay. To examine the effect of FLL, OA and UA on the apoptosis of osteoclasts, the RAW 264.7 cells were seeded into 60-mm cell culture dishes at a density of 1x10⁵ cells per dish, stimulated with RANKL and cultured for 5 days. Following this, various concentrations of FLL, OA and UA were added into the medium for a further 2 days. Subsequently, the cells were treated with trypsin without EDTA, harvested and stained with

Table I. Effect of FLL, OA and UA on TRAP activity in RANKL-induced RAW264.7 cells.

Treatment	Concentration (µg/ml)	TRAP activity (%)	P-value
FLL	0	100.0±10.4	/
FLL	10	71.2±8.1	1.000
FLL	25	66.6±1.6	0.006
FLL	50	70.5±4.4	0.007
FLL	100	76.1±6.1	0.008
OA	0	100.0±10.4	/
OA	0.36	51.0±5.3	0.939
OA	0.89	48.4±5.3	4.086x10 ⁻⁸
OA	1.78	55.7±8.9	1.616x10 ⁻⁸
OA	3.56	60.8±15.0	5.527x10 ⁻⁸
UA	0	100.0±10.4	/
UA	0.10	54.2±16.2	1.000
UA	0.26	44.0±3.3	0.014
UA	0.52	41.3±4.7	2.743x10 ⁻⁴
UA	1.03	45.4±5.9	1.395x10 ⁻⁴

Control cells were cultured with RANKL alone. Data are expressed as TRAP activity percentage (average TRAP activity/average TRAP activity of control). Data are presented as the mean ± standard deviation (n=5). Significant differences were observed between all the treated groups and control. FLL, *Fructus ligustri Lucidi*; OA, oleanolic acid; UA, ursolic acid; TRAP, tartrate-resistant acid phosphate; RANKL, receptor activator of nuclear factor κB ligand.

an Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Detection kit (Invitrogen; Thermo Fisher Scientific, Inc.). Finally, the cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed by CellQuest Pro version 5.1 (BD Biosciences).

RNA extraction. Total RNA was isolated from cells treated with 50 ng/ml RANKL and FLL, OA and UA using TRIzol® reagent (Beijing Biotides Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's protocol. The quality and quantity of the extracted mRNA were assessed by spectrophotometry, with the ratios of absorbance at 260 and 280 nm ranging from 1.9 to 2.0.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). cDNA was synthesized with a RT system kit (Promega Biotech Co., Ltd., Beijing, China) and stored at -20°C until further processing. Expression of selected genes was measured by qPCR using a Bio-Rad CFX9600 system (Bio-Rad Laboratories, Inc.) with the Maxima SYBR® Green qPCR Master Mix (2X; Invitrogen; Thermo Fisher Scientific, Inc.). Primer sequences were as follows: For TRAP, forward 5'-GAACCGTGCAGACGATGG-3' and reverse 5'-GGAAGT TCCAGCGCTTGG-3'; for CTSK, forward 5'-CTGCCATA ACCTGGAGG-3' and reverse 5'-GCCCTGGTTCTTGAC TGG-3'; for MMP-9, forward 5'-GGTCTAGGCCAGAG

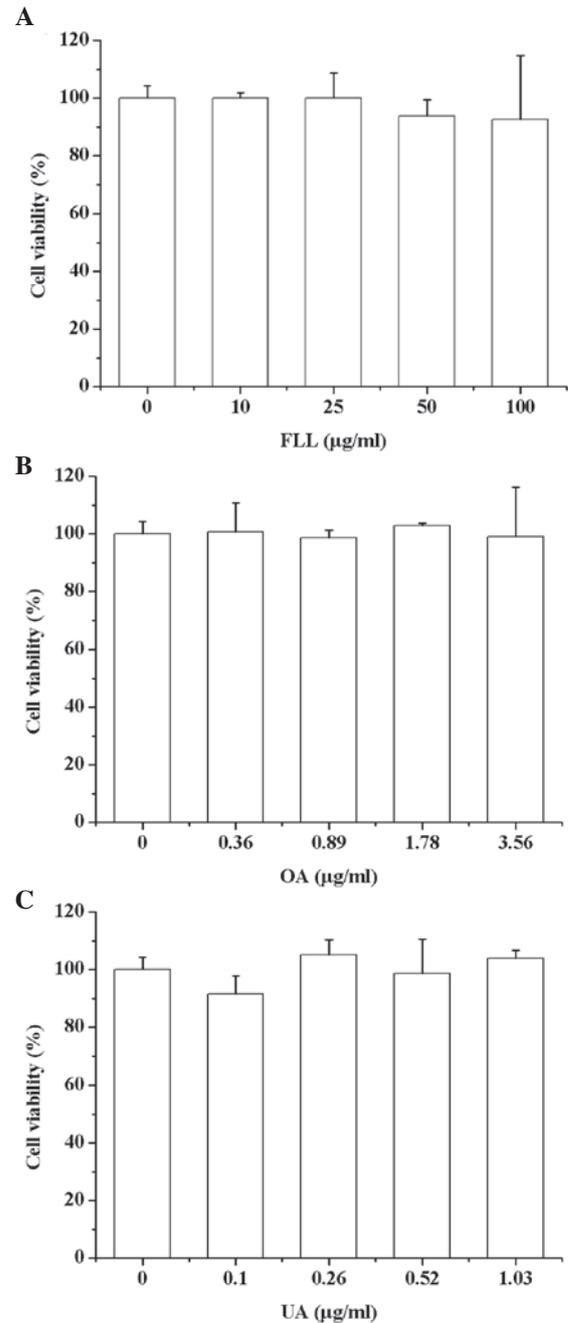


Figure 1. Effect of FLL, OA and UA on RAW264.7 cell viability. Effect of (A) FLL, (B) OA and (C) UA on RAW264.7 cell viability, as measured by MTT assay. The data are presented as cell viability percentage (average OD/average control OD). All data are expressed as the mean ± standard deviation (n=3). No statistically significant differences were observed. FLL, *Fructus ligustri Lucidi*; OA, oleanolic acid; UA, ursolic acid; OD, optical density.

GTA-3' and reverse 5'-GGTCGTAGGTCACGTAGC-3'; for TRAF-6, forward 5'-ATTCTCGACCAGTCTGAAG-3' and reverse 5'-ATGAAGGTTCCCTGTCT-3'; for NFAT-c1, forward 5'-TCATCCTGTCCAACACAAA-3' and reverse 5'-TCACCC TGGTGTCTTCCTC-3'; for c-Fos, forward 5'-CGAAGG GAACGGAATAAGAT-3' and reverse 5'-GCAACGCAGACT TCTCATC-3'; for Src, forward 5'-TCGTGAGGGAGAGTG AGAC-3' and reverse 5'-GCGGGAGGTGATGTAGAAAC-3'; for GAPDH, forward 5'-AACTTTGGCATTGTGGAAGG-3'

Table II. Effect of FLL, OA and UA on osteoclast apoptosis.

Treatment	Concentration ($\mu\text{g/ml}$)	Apoptosis rate (%)	
		Early stage	Late stage
FLL	0	5.4 \pm 3.3	2.1 \pm 1.4
FLL	10	5.5 \pm 2.0	2.1 \pm 1.3
FLL	25	6.6 \pm 4.5	2.5 \pm 1.2
FLL	50	5.6 \pm 3.0	2.8 \pm 1.8
FLL	100	6.8 \pm 3.8	3.1 \pm 1.7
OA	0	5.4 \pm 3.3	2.1 \pm 1.4
OA	0.36	6.6 \pm 3.5	2.9 \pm 1.2
OA	0.89	4.8 \pm 1.9	2.6 \pm 1.2
OA	1.78	6.4 \pm 3.2	2.6 \pm 1.2
OA	3.56	5.0 \pm 2.0	2.8 \pm 1.5
UA	0	5.4 \pm 3.3	2.1 \pm 1.4
UA	0.10	6.0 \pm 2.1	2.3 \pm 1.5
UA	0.26	6.0 \pm 3.7	2.6 \pm 1.6
UA	0.52	4.9 \pm 4.3	2.2 \pm 1.8
UA	1.03	4.7 \pm 2.5	2.1 \pm 0.7

Cells were stained with annexin V-FITC and PI. Early apoptotic cells were defined as annexin V-FITC⁺/PI⁻; late apoptotic cells were defined as annexin V-FITC⁺/PI⁺. Data are presented as the percentage of apoptotic cells and all data are presented as the mean \pm standard deviation (n=3). No significant differences were observed compared with the control group. FLL, *Fructus ligustri Lucidi*; OA, oleanolic acid; UA, ursolic acid; FITC, fluorescein isothiocyanate; PI, propidium iodide.

and reverse 5'-ACACATTGGGGGTAGGAACA-3'. For each primer pair, the analysis was performed in triplicate with a total volume of 25 μl reaction mixture. The cycling conditions for PCR were as follows: An initial denaturation step of 10 min at 95°C, followed by 45 cycles of denaturation at 94°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 20 sec. Melting curves were assessed to ensure the amplification was specific. Relative gene expression was determined by employing the formula $2^{-\Delta\Delta C_t}$ (26). mRNA expression levels in all experimental groups were compared with those of the control group.

Statistical analysis. All data are expressed as the mean \pm standard deviation. At least 3 independent experiments were conducted in duplicate. Statistical analysis was performed using SPSS software version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). One-way analyses of variance were used to identify differences between values of experimental and control groups. All identified differences were tested for homogeneity of variances, if equal variance was indicated, followed by the least significant difference test (LSD), or Tamhane's T2 test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of the FLL extract. Peaks of OA and UA appeared at 24.582 and 26.093 min, respectively, in HPLC, compared with the retention times of the standard solutions, 24.5 min for OA

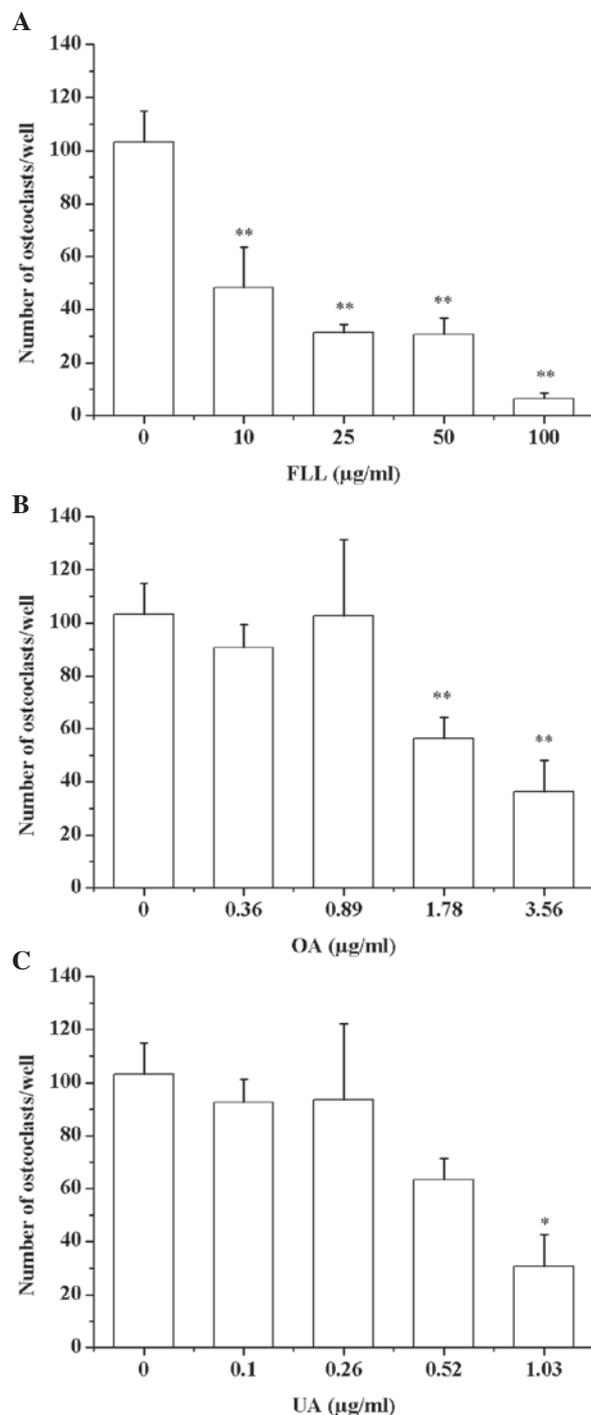


Figure 2. Numbers of TRAP-positive multinucleate osteoclasts following FLL, OA or UA treatment. Multinucleate (nuclei, ≥ 3) osteoclasts in (A) FLL, (B) OA and (C) UA treatment groups were counted. The data are presented as the number of osteoclasts per well. All data are expressed as the mean \pm standard deviation (n=3). Control cells were cultured with RANKL alone. All concentrations of FLL and high concentrations of OA and UA reduced the number of osteoclasts observed. * $P < 0.05$ and ** $P < 0.01$ vs. control group. FLL, *Fructus ligustri Lucidi*; OA, oleanolic acid; UA, ursolic acid; TRAP, tartrate resistant acid phosphatase; RANKL, receptor activator of nuclear factor κB ligand.

and 26.0 min for UA. Using the external standard method, the contents of OA and UA in FLL were quantified as 3.56 and 1.03%, respectively. The present study selected the concentrations of 10, 25, 50 and 100 $\mu\text{g/ml}$ FLL on the basis of a previous

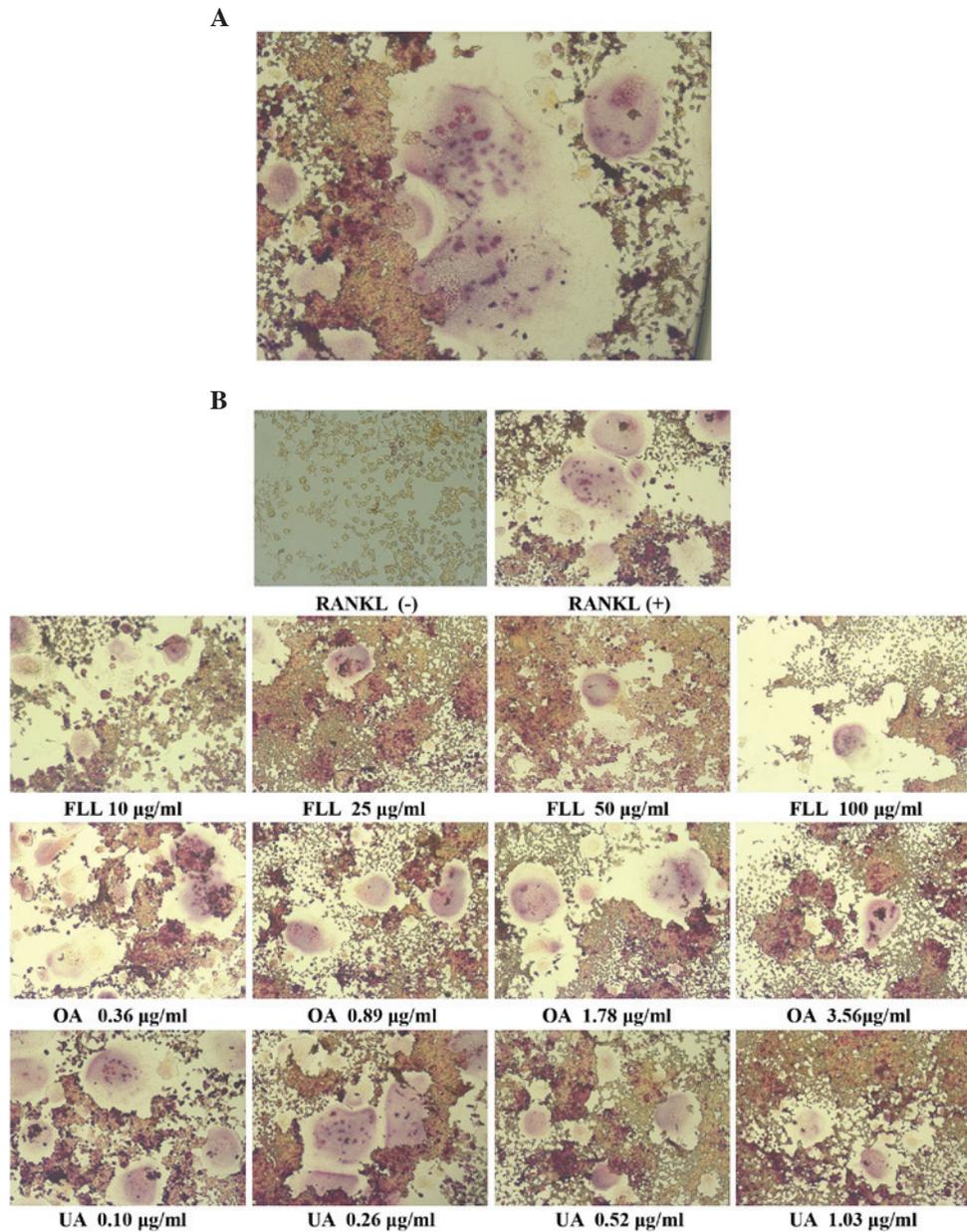


Figure 3. Images of TRAP stained osteoclasts. Images were captured with a light microscope (magnification, x100) and the TRAP-positive multinucleate (nuclei, ≥ 3) osteoclasts appeared purple or dark red. (A) Image of a unique TRAP-positive multinucleate osteoclast. This mature osteoclast had multiple nuclei within an osteoclast. The nuclei appeared colorless and the cytoplasm appeared dark red. (B) Images of TRAP-positive multinucleate osteoclasts following FLL, OA or UA treatment. FLL, *Fructus ligustri Lucidi*; OA, oleanolic acid; UA, ursolic acid; RANKL, receptor activator of nuclear factor κ B ligand; TRAP, tartrate resistant acid phosphatase.

study about the effect of FLL ethanol extract on osteoblast-like cell line (22). Based on the quantity of OA and UA in FLL, the concentrations of 0.36, 0.89, 1.78 and 3.56 µg/ml OA, and 0.10, 0.26, 0.52 and 1.03 µg/ml UA were selected.

FLL, OA and UA are not cytotoxic to RAW264.7 cells. As presented in Fig. 1, no concentration of the FLL extract (10, 25, 50 and 100 µg/ml), OA (0.36, 0.89, 1.78 and 3.56 µg/ml) or UA (0.10, 0.26, 0.52 and 1.03 µg/ml) induced cytotoxicity in RAW264.7 cells compared with the control group.

FLL, OA and UA inhibit RANKL-induced osteoclastogenesis of RAW264.7 cells. To examine the effect of FLL, OA and UA on osteoclast differentiation, the RANKL-treated cells

were stained for TRAP and TRAP activity was assessed. In the TRAP activity assay (Table I), significant differences were observed in all the FLL, OA and UA treated groups except in the lowest concentration groups, but not in a dose-dependent manner. Regarding TRAP staining, FLL significantly inhibited the osteoclastogenesis of RAW264.7 cells in a dose-dependent manner. The number of osteoclasts per well declined with increasing FLL concentration, and significant differences were identified between all treated groups and the control group (Fig. 2A; $P < 0.01$). However, in OA- and UA-treated groups significant differences were identified only in the high concentration groups (1.78 µg/ml OA vs. Control group: $P = 0.007$; 3.56 µg/ml OA vs. Control group: $P = 0.001$; and 1.03 µg/ml UA vs. Control group: $P = 0.016$ compared with

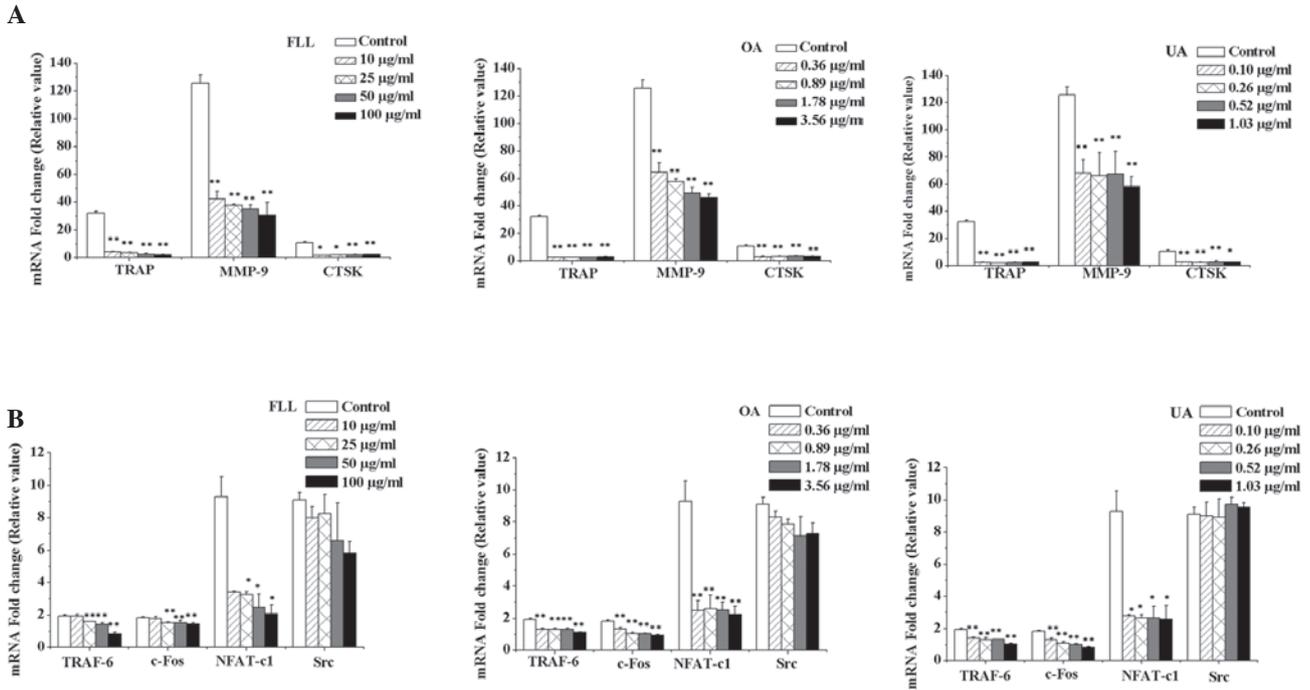


Figure 4. mRNA expression levels of osteoclast-associated genes. Reverse transcription-quantitative polymerase chain reaction was performed to determine the effect of FLL, OA and UA on the mRNA expression levels of osteoclast-associated genes. (A) Effect of FLL, OA and UA on the mRNA expression levels of the osteoclast markers, TRAP, MMP-9 and CTSK in RAW264.7 cells stimulated with RANKL. All concentrations of FLL, OA and UA reduced the mRNA expression levels of all three genes. (B) Effect of FLL, OA and UA on the mRNA expression levels of the signaling-associated genes, TRAF-6, c-Fos, NFAT-c1 and Src in RAW264.7 cells stimulated with RANKL. FLL, OA and UA reduced the mRNA expression levels of TRAF-6, c-Fos and NFAT-c1; however, no effect on Src was observed. The mRNA fold change value of TRAP, MMP-9, CTSK, TRAF-6, c-Fos, NFAT-c1 and Src in RAW264.7 cells were all set as 1. Control cells were cultured with RANKL alone. All data were presented as the mean \pm standard deviation ($n=3$). * $P<0.05$ and ** $P<0.01$ vs. the control group. FLL, *Fructus ligustri Lucidi*; OA, oleanolic acid; UA, ursolic acid; RANKL, receptor activator of nuclear factor κ B ligand; TRAP, tartrate resistant acid phosphatase; CTSK, cathepsin K; MMP-9, matrix metalloproteinase-9; TRAF-6, tumor necrosis factor receptor associated factor-6; NFAT-c1, nuclear factor of activated T cell-c1.

the control group (Fig. 2B and C). Fig. 3 presented the images of TRAP stained cells. The TRAP-positive multinucleate cells appeared dark red and contained ≥ 3 nuclei, whereas the RAW264.7 cells without RANKL treatment appeared brown. The number of multinucleate osteoclasts observed decreased with increasing concentrations of FLL, OA and UA.

FLL, OA and UA demonstrated no effect on apoptosis of osteoclasts. As presented in Table II, FLL (10, 25, 50 and 100 $\mu\text{g/ml}$), OA (0.36, 0.89, 1.78 and 3.56 $\mu\text{g/ml}$) and UA (0.10, 0.26, 0.52 and 1.03 $\mu\text{g/ml}$) demonstrated no effect on apoptosis of osteoclasts at the early (annexin V-FITC⁺/PI⁻) or late (annexin V-FITC⁺/PI⁺) stages compared with the control group.

RANKL-induced mRNA expression levels of osteoclast-associated genes were suppressed by FLL, OA and UA. To further examine the mechanisms underlying the effect of FLL, OA and UA on osteoclast differentiation, the present study investigated the mRNA expression levels of various osteoclast-associated genes. As presented in Fig. 4, FLL, OA and UA decreased the mRNA expression levels of osteoclast markers including TRAP, CTSK and MMP-9 (Fig. 4A). In addition, RANKL-induced expression of TRAF-6 (Fig. 4B), which is recruited following RANKL binding to RANK and induces downstream signaling pathways, was downregulated. Furthermore, RANKL-induced expression of key transcription

factors, including c-Fos and NFAT, was inhibited by FLL, OA and UA (Fig. 4B); however, no effect was observed on RANKL-induced expression of Src (Fig. 4B).

Discussion

Increased bone resorption by osteoclasts has a marked association with the occurrence of a large proportion of osteoporosis cases (27). Therefore, agents inhibiting osteoclast formation may make a notable therapeutic contribution to the treatment of osteoporosis (28). The present study was designed to clarify the effects of FLL on osteoclastogenesis *in vitro*, and the mechanisms underlying its effects. The results demonstrated that ethanol extract of FLL may inhibit RANKL-induced osteoclastogenesis in RAW264.7 cells without inducing cytotoxicity. In addition, the results suggested that OA and UA in FLL together account for its bioactive effects.

The results of the present study suggested that ethanol extract of FLL is an efficient but mild inhibitor of osteoclastogenesis. FLL extract and its two primary components, OA and UA, markedly suppressed RANKL-induced multinucleate osteoclast formation and TRAP activity simultaneously, without leading to cytotoxicity or affecting osteoclast apoptosis. Furthermore, RANKL-induced mRNA expression levels of TRAP, MMP-9 and CTSK, which are regarded as osteoclast markers, were all efficiently suppressed. The expression of these marker genes is associated with the terminal differentiation of

monocyte-macrophage lineage cells to osteoclasts (29,30), and allowed them to resorb bone matrix (31). TRAP and CTSK are lysosomal enzymes that contribute to osteoclast maturation (32) and bone matrix degradation (33), whereas MMP-9 is the most abundant MMP family member and is required for osteoclast migration (34).

In addition, the results of the present study suggested that nuclear factor κ B (NF- κ B)/mitogen-activated protein kinases (MAPK) may be associated with the FLL-mediated inhibition of osteoclastogenesis. mRNA expression levels of various key molecules of these signaling pathways were observed to be downregulated. Binding of RANKL to its receptor RANK recruits and induces the trimerization of the adaptor molecule TRAF-6 (7), thus leading to activation of MAPKs, phosphatidylinositol 3-kinase and NF- κ B (8-10). TRAF-6-deficient mice have marked levels of osteoporosis and inhibited osteoclast formation (35). The results of the present study suggested that RANKL-induced assembly of TRAF-6 may be limited by FLL, OA and UA, and consequently suppress downstream signaling via TRAF-6. Furthermore, it was observed that transcription factors including c-Fos and NFAT-c1 may be involved. c-Fos is essential for osteoclast differentiation and c-Fos-deficient mice develop osteoporosis (36). NFAT-c1, activated by NF- κ B, is considered a key regulator of RANKL-induced osteoclast differentiation, fusion and activation (30,37). Furthermore, the activity of NFAT-c1 is enhanced by the overexpression of c-Fos (38), indicating that cooperation between c-Fos and NFAT-c1 may be important for osteoclast formation and activation. The present study demonstrated that FLL, OA and UA inhibited RANKL-induced expression of c-Fos and NFAT-c1, thereby attenuating osteoclastogenesis. Notably, the present study did not observe any downregulation of RANKL-induced expression of Src. Src is involved in osteoclast differentiation (39) and activation of Src family members regulated the activation of the anti-apoptotic protein kinase B (40). Thus expression and activation of Src may be associated with mortality. Osteoclasts from Src-deficient mice failed to form ruffled borders and resorption lacunae (41). This evidence suggests that FLL is a mild agent, as it did not demonstrate an effect on Src and did not promote apoptosis in the present study.

However, interactions of OA and UA in FLL remain to be elucidated. Concentrations of OA and UA in the present study were selected according to their proportion in FLL, to understand their specific roles in the bioactive effects of FLL. The results suggested that a combination of OA and UA accounted for the effect of FLL on osteoclastogenesis inhibition, as they inhibited osteoclastogenesis independently. However, the effects of FLL, OA and UA on osteoclastogenesis and mRNA expression levels were not identical. For example, at the lowest concentration (0.36 μ g/ml for OA and 0.10 μ g/ml for UA), the two components suppressed mRNA expression levels of NFAT-c1; however, the lowest concentration of FLL (10 μ g/ml) appeared to exert no effect. These inconsistencies suggested complex interactions between OA and UA in FLL. OA and UA exhibit various biological activities, including anti-inflammatory, anti-cancer and hepatoprotective effects (42,43). Additionally, there are various other ingredients in FLL that may function in this specific process, including linoleic acid, salidroside and acetyl oleanolic acid (44). Thus, further investigation is necessary to fully elucidate the underlying mechanisms.

In conclusion, the results of the present study demonstrated that ethanol extract of FLL may inhibit osteoclastogenesis in RAW264.7 cells via RANKL signaling pathways. The results suggested that the number of RANKL-induced osteoclasts and their bone-resorption abilities were reduced in the presence of ethanol extract of FLL. OA and UA were demonstrated to be active components in FLL; however, their exact underlying mechanism of action and any potential interactions between them remain to be elucidated. FLL is now a compound of interest for the prevention and treatment of osteoporosis, and the results of the present study indicated that OA and UA may be used instead of FLL for future treatments.

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