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RESEARCH

In vitro effects of ursolic acid on RANKL-induced osteoclast differentiation

Ursolik asidin RANKL ile indüklenen osteoklast farklılaşması üzerindeki *in vitro* etkileri

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Abstract

Purpose: Ursolic acid is a small pentacyclic triterpene molecule composed of isoprenoid units. Although ursolic acid has been shown to be effective in the treatment of rheumatoid arthritis in many studies, very little research has been conducted on bone diseases caused by bone loss. The present study aimed to evaluate the effects of ursolic acid on osteoclast formation with the aim of finding herbal medicines that inhibit osteoclast function to strengthen bones and promote vitality in old age.

Materials and Methods: RAW264.7 murine macrophages were used in our study and cells were treated with 100 ng/mL RANKL for osteoclastic differentiation. The effects of ursolic acid treatment on cell viability, tartrate-resistant acid phosphatase (TRAP) formation and osteoclastic gene expression levels were then measured.

Results: Our results showed that ursolic acid did not exhibit significant cytotoxicity (3.2-9.8%) at concentrations of 2.5-10 µg/mL. Furthermore, ursolic acid inhibited osteoclast differentiation (15.2-39.1%) and suppressed the expression of osteoclastic genes such as *cathepsin K* (3.8-22.3%), *TRAP* (16.3-48. 7%), matrix metalloproteinase-9 (*MMP-9*) (10.7-40.2%), nuclear factor of activated T-cell cytoplasmic 1 (*NFATc1*) (1.2-29.7%), *c-Fos* (0.9-13.8%) and microphthalmia-associated transcription factor (*MITF*) (2.2-21.6%).

Conclusion: Ursolic acid has been shown to inhibit RANKL-induced osteoclast differentiation and therefore we believe that ursolic acid may be used for the treatment and prevention of osteoporosis.

Keywords:. Ursolic acid, RAW264.7 macrophages, osteoporosis, osteoclast, RANKL

Öz

Amaç: Ursolik asit, izoprenoid birimlerden oluşan küçük bir pentasiklik triterpen molekülüdür. Ursolik asidin romatoid artrit tedavisinde etkili olduğu birçok çalışmada gösterilmiş olmasına rağmen, kemik kaybının neden olduğu kemik hastalıkları üzerine çok sınırlı araştırma yapılmıştır. Bu çalışma, kemikleri güçlendirmek ve yaşlılıkta canlılığı artırmak için osteoklast fonksiyonunu inhibe eden bitkisel ilaçlar bulmak amacıyla ursolik asidin osteoklast oluşumu üzerindeki etkilerini değerlendirmeyi amaçlamıştır.

Gereç ve Yöntem: Çalışmamızda RAW264.7 fare makrofajları kullanılmış ve hücreler osteoklastik farklılaşma için 100 ng/mL RANKL ile muamele edilmiştir. Daha sonra, ursolik asit tedavisinin hücre canlılığı, tartarat dirençli asit fosfataz (TRAP) oluşumu ve osteoklastik gen ekspresyon seviyeleri üzerindeki etkileri ölçülmüştür.

Bulgular: Sonuçlarımız ursolik asidin 2.5-10 µg/mL konsantrasyonlarında önemli bir sitotoksisite sergilemediğini göstermiştir (%3,2-9,8). Ayrıca, ursolik asit osteoklastların farklılaşmasını inhibe etmiş (%15,2-39,1) ve *katepsin K* (%3,8-22,3), *TRAP* (%16,3-48,7), matriks metalloproteinaz-9 (*MMP-9*) (%10,7-40,2), aktive T hücre sitoplazmik nükleer faktörü-1 (*NFATc1*) (%1,2-29,7), *c-Fos* (%0,9-13,8) ve mikroftalmi ile ilişkili transkripsiyon faktörü (*MITF*) (%2,2-21,6) gibi osteoklastik genlerin ekspresyonunu baskılamıştır.

Sonuç: Ursolik asidin RANKL ile indüklenen osteoklast farklılaşmasını inhibe ettiği gösterilmiştir ve bu nedenle ursolik asidin osteoporozun tedavisi ve önlenmesi için kullanılabileceği kanaatini taşımaktayız.

Anahtar kelimeler: Ursolik asit, RAW264.7 makrofajları, osteoporoz, osteoklast, RANKL

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INTRODUCTION

As mammals, including humans, age, their ability to regulate hormone secretion changes, leading to a range of changes in physical activity. Age-related diseases such as diabetes, osteoporosis, hypertension and hyperlipidaemia, arthritis and obesity can be significantly reversed by maintaining hormonal balance. However, the chronic nature of most geriatric conditions makes adequate treatment difficult due to lack of consistent attention and limited availability of hormone therapy[1,2].

Osteoporosis, a weakening of the bones, is a common geriatric condition whose prevalence increases with age, similar to thyroid nodules and prostate enlargement, and is known to cause fractures, which are the leading cause of death in the elderly. Bone weakness leads to loss of mobility in both humans and animals, and secondarily shortens life span by causing or exacerbating other geriatric conditions. Therefore, strengthening bones to prevent or treat bone-related diseases is essential for maintaining health in old age[2].

In mammals, bone health is maintained by the regulation of calcium metabolism in bone by osteoblasts, which build bone tissue, and osteoclasts, which break down bone tissue. In healthy bones, both processes are balanced to maintain bone tissue, but when osteoclast function becomes dominant over osteoblast function, osteoporosis with bone loss occurs. Therefore, osteoclast differentiation and inhibition of differentiated osteoclast function are necessary to maintain bone tissue in a healthy state[3-5].

Osteoclast differentiation is initiated when a cytokine called receptor activator of nuclear factor kappa-B ligand (RANKL), secreted upon activation of osteoblast function, forms a complex with RANK present on osteoclast precursors[6]. Activation of tumour necrosis factor receptor-associated factor (TRAF) signals extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, while signals to transcriptional regulators such as nuclear factor of activated T-cell cytoplasmic 1 (NFATc1) and nuclear factor-kappa B (NF-κB) regulate the osteoclast differentiation process[7-9]. This differentiation process, including the action of dendritic cell-specific transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP), increases osteoclast maturation, migration, fusion and cell viability through apoptosis inhibitory mechanisms[10]. Through this sequence of events, differentiated osteoclasts express the biosynthesis of bone resorption proteins such as tartrate-resistant acid phosphatase (TRAP), cathepsin K and matrix metalloproteinase-9 (MMP-9), ultimately leading to bone resorption. In addition, it has been reported that bone tissue damage caused by bone resorption is associated with activation of osteoclast function through activation of inflammation-related factors[11]. Therefore, many studies have been conducted to inhibit bone resorption and strengthen bone by inhibiting osteoclast differentiation and function. In recent years, studies have focused on various plant extracts and phytochemicals that inhibit osteoclast function to strengthen bones and increase bone vitality, which declines with age[2]. Triterpenoids are the largest and most structurally diverse group of terpenoids and are synthesised from squalene or related 30-carbon precursors. Most triterpenoids have 30 carbons and consist of the structural composition of six isoprene units. Triterpenoids are divided into two groups: tetracyclic triterpenoids and pentacyclic triterpenoids. The most important known pentacyclic triterpenoids are boswellic acid, betulinic acid and ursolic acid. Pentacyclic triterpenoids have many important pharmacological and biological effects such as anti-hyperglycemic, hepatoprotective, immunomodulatory, antioxidant, anti-inflammatory and anti-tumour activities. The lupan, olean and ursan subgroups of pentacyclic triterpenoids are responsible for these activities[12,13].

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is a small pentacyclic triterpene molecule composed of isoprenoid units. *Rosmarinus officinalis*, *Ocimum basilicum*, *Eriobotrya japonica, Eugenia jambolana*, *Origanum vulgare*, *Eucalyptus globulus*, *Coffea arabica* and *Pyrus malus* are the main plants known to contain ursolic acid[12]. Ursolic acid has low toxicity and multiple biological activities. Ursolic acid has remarkable pharmacological properties. The most important of these are antihyperlipidemic, hepatoprotective, antitumoral, sedative, anti-ulcer, antibacterial, antiprotozoal, anti-HIV, anti-obesity, anti-diabetic, immunomodulatory and analgesic activities[13]. The stimulatory effect of ursolic acid on oxidative and inflammatory damage was also investigated. As a result of the study, a reduction in both types of damage was observed and it was found that the viability of the cells studied increased significantly. In particular, ursolic acid has been reported to inhibit the action of MMP-9 by inhibiting

NF-kB[14]. In vitro studies have shown that ursolic acid stimulates osteoblast differentiation and mineralisation by activating mitogen-activated protein (MAP) kinases and transcription factors[15].

These data suggest that ursolic acid may inhibit osteoclast differentiation or function[15]. This study hypothesises that ursolic acid may inhibit osteoclast differentiation or function. The effect of ursolic acid on osteoclastic activity and its specific mechanism of action have not been addressed in the literature, and mechanistic studies on this topic are lacking. The aim of this study was to evaluate the effects of ursolic acid on osteoclast formation in order to identify herbal remedies that inhibit osteoclast function to strengthen bones and increase vitality in old age. To this end, TRAP-positive formation capacity was measured in RANKL-stimulated RAW264.7 cells and osteoclastogenesis-related gene expression levels were measured. The results of this study may provide valuable information to the literature and to researchers working in the field of osteoporosis treatment and prevention.

MATERIALS AND METHODS

Cell culture

This study was designed as an in vitro study in September 2023. This research was conducted in the laboratories of the Central Research Laboratory and the Faculty of Pharmacy of Van Yüzüncü Yıl University. The university's state-of-the-art laboratories and extensive research facilities allow for comprehensive studies in a variety of scientific disciplines.

RAW264.7 cells, a transformed macrophage cell line derived from the BALB/c mouse, are well characterised and widely used in cell culture studies. Therefore, RAW264.7 macrophage cells were kindly provided by Prof Dr Ahmet Cumaoğlu (Erciyes University Faculty of Pharmacy). Ethical committee approval is not required as a commercial cell line was used in the study. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L D-glucose, L-glutamine, sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C. RAW264.7 cells were seeded in 24-well plates at $1x10^4$ cells per well. After 24 hours of incubation, the cells were cultured for 5 days in DMEM supplemented with 100 ng/mL RANKL (Sigma-Aldrich, St Louis, MO) for 4 days to induce differentiation. The medium was changed every 1-2 days during the culture period.

Cell viability assay

The viability of RAW264.7 cells was assessed using the MTT assay. Ursolic acid (Sigma-Aldrich Chemical Co, St. Louis, USA, catalogue number 68923) was dissolved in dimethyl sulfoxide (DMSO) and diluted to working concentrations with DMEM. The final concentration of DMSO in the medium for all cell-based experiments was 0.1%. Cells were seeded on 96-well plates at a density of 1×10^4 cells/well for 24 hours and exposed to different concentrations of ursolic acid (2.5-100 mg/mL) in the absence or presence of RANKL (100 ng/mL)[16]. After 24 hours of incubation, the medium was removed and 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was diluted with the medium, and 100 µL of 0.5 µg/mL

MTT solution was added to each well and incubated for 4 hours. The MTT solution was completely removed and 100 µL DMSO was added to dissolve the formazan formed in each well and the reaction was incubated for 10 minutes at room temperature. Absorbance was measured at 540 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The mean absorbance value of the untreated group was calculated and expressed as a percentage of the mean absorbance value of the ursolic acid treated group and expressed as cell viability[17].

TRAP Staining

Osteoclast differentiation was induced by treatment of RAW 264.7 cells with RANKL and assessed by staining for TRAP as a differentiation marker expressed in differentiated osteoclasts and identification of TRAP-positive multinucleated cells. Differentiated cells were washed twice with phosphate-buffered saline, followed by fixation with 3.7% formaldehyde-citrate-acetone solution for 10 minutes, and then washed again twice with distilled water.

The fixed cells were washed twice with distilled water and reacted with the TRAP staining kit (Sigma-Aldrich, St Louis, MO) for at least 1 hour at room temperature. TRAP-positive multinucleated cells were counted using an Olympus BX43 light microscope[18].

Estimation of mRNA levels

To assess osteoclastic gene expression, RNA was extracted and gene expression was determined by real-time polymerase chain reaction (RT-PCR). RNA was isolated by adding Trizol agent to the cells, and 1 μg of RNA was used for cDNA synthesis. RT-PCR analysis was performed using Invitrogen Universal EXPRESS SYBR GreenER quantitative PCR SuperMixes and Two-Step quantitative RT-PCR Kit. mRNA levels were quantified by RT-PCR using a Bio-Rad CFX Connect Real Time PCR Detection System device (Bio-Rad Laboratories, Hercules, CA, USA). Primer information used in this experiment is shown in Table 1[19]. The expression of each gene was quantified relative to β-actin expression and results were expressed as fold change of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences and conditions for RT-PCR[19]

Target gene	Forward Primer	Reverse Primer	Anneling Tm (°C)	PCR cycles
TRAP	5'-ctgctgggcctacaaatcat-3'	5'-ggtagtaagggctggggaag-3'	54	30
MMP-9	5'-cgtcgtgatccccacttact-3'	5'-agagtactgcttgcccagga-3'	57.5	36
Cathepsin K	5'-aggcggctatatgaccactg-3'	5'-ccgagccaagagagcatatc-3'	57.5	26
NFATc1	5'-gggtcagtgtgaccgaagat-3'	5'-aggtgggtgaagactgaagg-3'	55	35
c-Fos	5'-atgggctctcctgtcaacac-3'	5'-ggctgccaaaataaactcca-3'	57.5	30
MITF	5'- agacctgacatgtacgacaac-3'	5'- tatgcagggctactgataag -3'	56	35
β-actin	5'-ttctacaatgagctgcgtgt-3'	5'-ctcatagctcttctccaggg-3'	50	26

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA). Results are presented as mean ± standard deviation. The Shapiro-Wilk test was used to check that the data conformed to a normal distribution. For MTT cytotoxicity analyses, Kruskall-Wallis analysis of variance was used to assess the difference between groups. For TRAP-positive osteoclast cell counting and gene expression analysis, statistical significance between each experimental group was determined using one-way ANOVA, followed by Tukey's multiple range test as a post hoc test. Results for mRNA levels were expressed as fold change in threshold cycle (Ct) relative to controls using the $2^{-\Delta\Delta Ct}$ method. Values $p<0.05$ were considered significant from the results obtained in the statistical analysis.

RESULTS

Cell viability

The effect of ursolic acid on the viability of RAW264.7 cells was investigated using the MTT assay. As shown in Figure 1, all concentrations of ursolic acid showed no significant difference in viability compared to intact RAW264.7 cells. On the other hand, in RANKL-treated RAW264.7 cells, only 2.5-10 μg/mL concentrations of ursolic acid caused negligible cell death, while 25-100 concentrations of ursolic acid caused cytotoxicity above 10% (Figure 1). Therefore, only 2.5, 5 and 10 μg/mL concentrations of ursolic acid were used in the further stages of our study.

Inhibition of RANKL-induced osteoclastogenesis

The effect of ursolic acid was studied during the differentiation of RAW264.7 cells into osteoclasts stimulated with RANKL. Osteoclast formation was monitored by observing the formation of giant multinucleated cells and by measuring the activity of TRAP, an osteoclast marker enzyme. The control group without RANKL treatment showed little TRAP expression, which is characteristic of osteoclast differentiation, but TRAP expression was significantly increased in the RANKL group compared to the control group. Treatment with ursolic acid showed an inhibitory effect of about 15.4% at a concentration of 2.5 μg/mL, about 27.7% at a concentration of 5 μg/mL and about 38.3% at a concentration of 10 μg/mL on the formation of TRAP-positive multinucleated cells induced by RANKL ($p<0.05$) (Figure 2A and 2B).

Expression of osteoclastogenesis-related genes

TRAP, *MMP-9*, *cathepsin K*, *NFATC1*, *c-FOS* and microphthalmia-associated transcription factor

(*MITF*) mRNA levels were significantly increased in the RANKL-treated group compared to the control group (p<0.05). *TRAP* and *MMP-9* gene expression in RANKL-induced RAW264.7 cells was found to be statistically significantly lower at all ursolic acid concentrations used in the study (p<0.05). *NFATC1* expression in RANKL-induced RAW264.7 cells was found to be statistically significantly lower only at 5 and 10 µg/mL ursolic acid concentrations (p<0.05). *c-FOS*, *cathepsin K* and *MITF* mRNA levels in RANKL-induced RAW264.7 cells were found to be statistically significantly lower at 10 µg/mL ursolic acid concentration (p<0.05) (Figure 3).

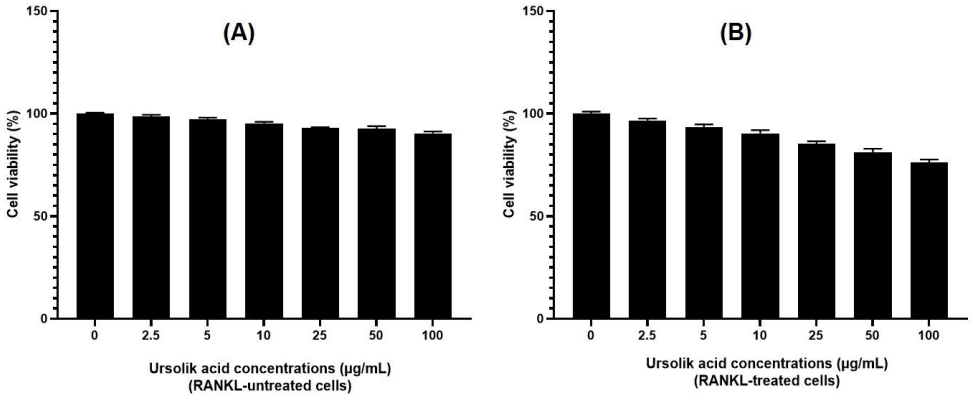

Figure 1. Effect of ursolic acid on cell proliferation of RAW264.7 cells. (A) RAW264.7 cells untreated with RANKL, (B) RAW264.7 macrophages treated with 100 ng/mL RANKL for 24h.

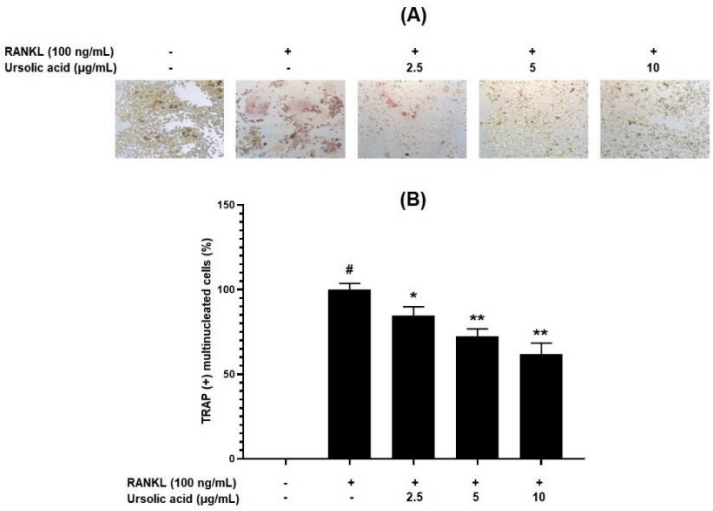

Figure 2. Effect of ursolic acid on RANKL-induced osteoclastogenesis. (A) TRAP-positive multinucleated cells were observed in RANKL-induced RAW264.7 macrophages under microscope, (B) Number of TRAP-positive osteoclasts.

[#] p<0.01; significantly different from untreated RAW264.7 macrophages
[*] p<0.05; significantly different from RANKL-induced RAW264.7 macrophages
[**] p<0.01; significantly different from RANKL-induced RAW264.7 macrophages

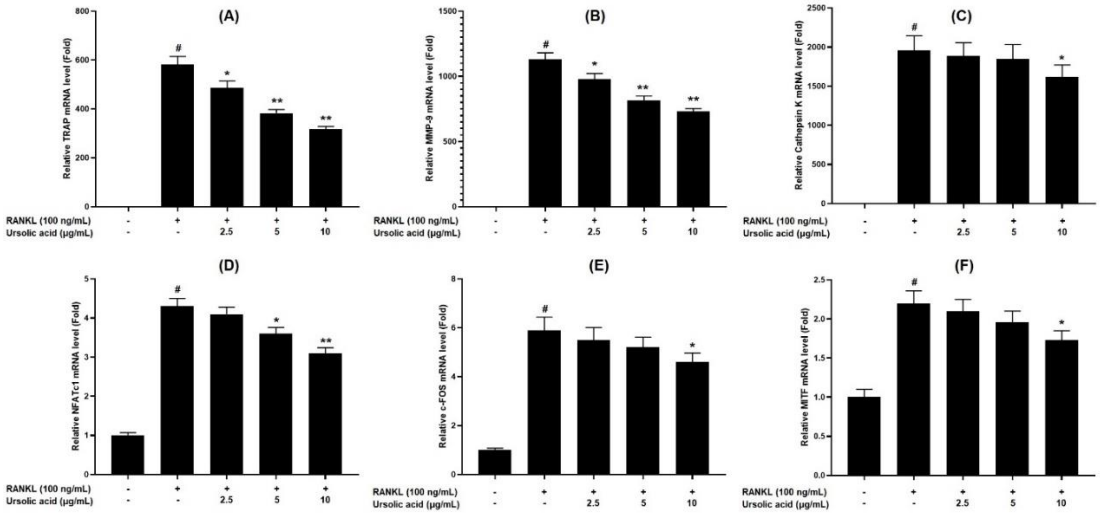

Figure 3. Effect of ursolic acid on osteoclastic gene expressions in RANKL-induced RAW264.7 macrophages. (A) *TRAP* mRNA level, (B) *MMP-9* mRNA level, (C) *cathepsin K* mRNA level, (D) *NFATc1* mRNA level, (E) *c-Fos* mRNA level, (F) *MITF* mRNA level.

$^{\#}$ $p<0.01$; significantly different from untreated RAW264.7 macrophages
* $p<0.05$; significantly different from RANKL-induced RAW264.7 macrophages
** $p<0.01$; significantly different from RANKL-induced RAW264.7 macrophages

DISCUSSION

Osteoporosis is a major cause of fractures, a major cause of death in the elderly, and can be caused by endocrine hormone abnormalities, drug side effects, nutrient deficiencies and genetic factors. Osteoporosis is an abnormality of the bone cells responsible for bone metabolism that results in rapid damage to the bone matrix and is characterised by the predominance of osteoclasts, which break down calcium-bound connective tissue in bone tissue to facilitate the release of calcium, over osteoblasts, which use calcium to build bone[20]. In addition to lifestyle changes, pharmacotherapy plays an important role in the treatment of osteoporosis. Current pharmacological treatments for osteoporosis include bone resorption inhibitors, bone formation stimulators and combination agents that inhibit bone resorption while stimulating bone formation. Bone resorption inhibitors include nutrients such as calcium and vitamin D, and hormonal agents such as estrogen, bisphosphonates, selective estrogen receptor modulators (SERMs) and calcitonin, while bone formation stimulators include sodium fluoride, parathyroid hormone and growth hormone. In addition, strontium is used as a combination of bone resorption inhibitor and bone formation stimulator. However, their limitations in the treatment of advanced osteoporosis, high cost and side effects such as breast cancer, venous thrombosis, stroke, gastrointestinal disorders and osteonecrosis of the jaw mean that further improvements are needed[21]. Therefore, there is an urgent need for research into the prevention and treatment of osteoporosis using herbal medicine approaches, with efforts being made to discover and develop natural product-based osteoclast inhibitors with few or no side effects[22]. Ursolic acid is a molecule that has long been used in folk medicine for the treatment of various diseases. This molecule is already known for its antioxidant, anti-inflammatory, antimicrobial and hepatoprotective effects[13]. Although ursolic acid has been shown in many studies to be effective in the treatment of rheumatoid arthritis, very little research has been done on bone diseases caused by bone loss[23]. Lee et al. suggested that ursolic acid has a preventive effect against osteoporosis by promoting bone formation, preventing bone resorption and reducing osteoclastic activity[24]. Ursolic acid may be a potential therapeutic agent for the treatment of osteoporosis due to its anabolic effects on bone metabolism[25]. Xiao et al. highlighted that ursolic acid

increases bone formation by activating MAP kinases and transcription factors such as NF-κB and AP-1[26]. Our study investigated the possible inhibitory effect of ursolic acid on osteoclast differentiation.

Osteoclasts are multinucleated cells of the monocyte/macrophage lineage derived from haematopoietic stem cells that are responsible for the destruction of bone tissue after differentiation and formation of osteoclast progenitors. Differentiation into osteoclasts is initiated by the action of differentiation-stimulating factors such as RANKL, a cytokine of the tumour necrosis factor family secreted predominantly by osteoblasts, as well as macrophage-colony stimulating factor (M-CSF)[27]. When osteoblast function is activated, osteoclast function is also activated to maintain homeostasis. RANK, which binds to RANKL, is present on the surface of osteoclast precursor cells. RANK is a member of the tumour necrosis factor receptor (TNFR) superfamily and when RANKL binds to it, it activates the MAPK pathway, including JNK, ERK and p38. This leads to the expression of transcription factors such as NFATc1, NF-κB, c-Fos and MITF, which are essential for osteoclast differentiation. Their action leads to the expression of bone matrix-degrading proteases such as TRAP, cathepsin K and MMP-9, which have a significant effect. On the other hand, activation of the ERK family of pathways inhibits osteoclast apoptosis and increases osteoclast function, while M-CSF interacts with RANKL, which promotes osteoclast differentiation, to inhibit apoptosis and maintain cell function[6,7,28].

In this study, we observed that ursolic acid inhibited osteoclast differentiation and evaluated its effects on the expression of differentiation-related factors. TRAP is a protein expressed in differentiated and mature osteoclasts and can be a reference marker to determine whether osteoclasts are differentiated and functionally active[29]. The results showed that ursolic acid inhibited the production of TRAP-positive multinucleated cells, which inhibited the formation of active cells through the differentiation process of osteoclasts.

Since TRAP is involved in bone resorption, the bone resorption process caused by the activation of osteoclasts was measured by the mRNA gene expression of *TRAP* in the cells. The results showed that RANKL increased *TRAP* expression in osteoclasts, and ursolic acid inhibited the RANKL-induced increase in *TRAP* expression, confirming that ursolic acid can inhibit the bone resorption role of osteoclasts[29]. This effect of ursolic acid was also seen on the expression of *MMP-9* and *cathepsin K*, another bone resorption factor responsible for osteoclast bone resorption. This led us to conclude that ursolic acid inhibits RANKL-induced protein synthesis in osteoclasts, which in turn inhibits bone resorption, as shown in the experimental results measuring bone resorption. Furthermore, this effect of ursolic acid was attributed to its inhibition of *NFATc1* expression, which regulates the expression of osteoclast-specific genes such as *MMP-9*, *TRAP*, and *cathepsin K*[30].

To determine the effect on the signalling system involved in the process of osteoclast differentiation and its relevance to the process of TRAP-positive multinucleated cell formation, we sought to evaluate the effect on the expression of transcription factors involved in the process of osteoclast differentiation[29]. c-Fos, NFATc1 and MITF are known to be key transcription factors that induce osteoclast differentiation. NFATc1 is a transcription factor whose expression is increased by TRAF activated by RANKL binding to its receptor, which in turn activates c-Fos and the calmodulin-activated kinase (CaMK) family of signalling pathways and plays an important role in the expression of factors required for osteoclast differentiation[31]. Park et al. showed that boswellic acid strongly inhibited RANKL-induced c-Fos and NFATc1 expression and attenuated the expression of osteoclast marker genes[32]. Jeong et al. showed that betulinic acid significantly suppressed osteoclastogenesis by decreasing Akt and IκB phosphorylation and PLCγ2-Ca^{2+} signalling in pathways involved in early osteoclastogenesis and subsequently suppressing c-Fos and NFATc1[33]. Wei et al. reported that betulinic acid administration prevented bone loss in ovariectomised mice and suppressed RANKL-associated osteoclastogenesis by inhibiting MAPK and NFATc1 pathways[34]. In our study, ursolic acid inhibited the expression of *NFATc1* and also inhibited the expression of *TRAP* and *cathepsin K*, whose expression is regulated by NFATc1 and which were predicted to be inhibited by ursolic acid. MITF, a transcription factor activated by TRAF signalling through TAK1, MKK6 and p38, is a transcription factor that promotes osteoclast differentiation[35]. In our experiments, ursolic acid reduced *MITF* expression, which was increased by RANKL, and also inhibited *c-Fos* expression, which would be expected to inhibit osteoclast differentiation from osteoblast precursors to osteoclasts[36].

The main limitation of this study is that the signalling pathways by which osteoclastogenesis-related genes are repressed were not shown. It was concluded that ursolic acid inhibits RANKL-induced osteoclast differentiation and activity by regulating the expression of various genes involved in osteoclast differentiation, thereby inhibiting bone resorption. Therefore, it is suggested that ursolic acid may be used for the treatment and prevention of osteoporosis. Future studies should investigate the therapeutic and prophylactic effects of ursolic acid on both postmenopausal osteoporosis and male pattern osteoporosis using experimental animal models and determine possible effects on different pathways on protein expression using Western blotting.

Author Contributions: Concept/Design : MB; Data acquisition: MB, MÇ; Data analysis and interpretation: MB, MÇ; Drafting manuscript: MB, MÇ; Critical revision of manuscript: MB, MÇ; Final approval and accountability: MB, MÇ; Technical or material support: MB; Supervision: MB; Securing funding (if available): n/a.
Ethical Approval: Since this research is a cell culture study and the RAW264.7 cell line is used; ethics committee approval is not required. The murine macrophage cell line (RAW264.7) used in this study was obtained from Prof. Dr. Ahmet CUMAOĞLU in Erciyes University Faculty of Pharmacy.
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