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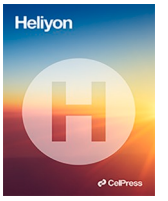

Research article

Trifolirhizin protects ovariectomy-induced bone loss in mice by inhibiting osteoclast differentiation and bone resorption

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ABSTRACT

Background: Osteoporosis is a debilitating condition characterized by reduced bone density and microstructure, leading to increased susceptibility to fractures and increased mortality, particularly among older individuals. Despite the availability of drugs for osteoporosis treatment, the need for targeted and innovative agents with fewer adverse effects persists. Trifolirhizin, a natural pterostalin derived from the root of Sophora flavescens, has been previously studied for its effects on certain anticancer and antiinflammatory. The impact of trifolirhizin on the formation and function of osteoclasts remain unclear.

Purpose: Herein, the possible roles of trifolirhizin the formation and function of osteoclasts and the underlying mechanism were explored. Methods: Bone marrow-derived macrophages (BMMs) were employed to evaluate the roles of trifolirhizin on steoclastogenesis, bone absorption and the underlying mechanism *in vitro*. Bone loss model was established by ovariectomy(OVX) in mice *in vivo*.

Results: Trifolirhizin repressed osteoclastogenesis, bone resorption induced by receptor activator of nuclear factor kappa B ligand (RANKL) *in vitro*. Mechanistically, trifolirhizin inhibits RANKL-induced MAPK signal transduction and NFATc1 expression. Moreover, trifolirhizin inhibited osteoclast marker gene expression, including *NFATc1, CTSK, MMP9, DC-STAMP, ACP5, and V-ATPase-D2*. Additionally, trifolirhizin was found to protect against ovariectomy(OVX)-induced bone loss in mice.

Abbreviations: LPS, lipopolysaccharide; OVX, ovariectomy; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand; MAPK, Mitogen-activated protein kinase; TF, trifolirhizin; TRAP, tartrate-resistant acid phosphatase; CTSK, cathepsin K; CTR, calcitonin receptor; MMP-9, matrix metallopeptidase 9; DMSO, Dimethyl sulfoxide; BMM, Bone marrow macrophage.

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Conclusion: Trifolirhizin can effectively inhibit osteoclast production and bone resorption activity. The results of our study provide evidence for trifolirhizin as a potential drug for the prevention and treatment of osteoporosis and other osteolytic diseases.

1. Introduction

Bone homeostasis is a process of continuous bone remodeling, mainly in which bone formation and bone resorption reach a dynamic equilibrium [1]. When the balance is broken, it is easy to lead to osteoporosis [2]. Patients with osteoporosis often have the phenotype of decreased bone mineral density, decreased bone strength and increased brittleness, resulting in an increased risk of bone fracture and death [3]. Clinical manifestations are usually brittle fractures, and statistics show that about 80 % of fractures are associated with osteoporosis [4].Therefore, it is crucial to actively look for prophylactic drugs that promote bone homeostasis and inhibit bone loss.

Bone resorption is mainly accomplished by osteoclasts, derived from monocytes, macrophages, and multinucleated cells [5]. The differentiation and formation of these cells rely on the activation of two specific cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) [6,7]. The combination of RANKL and RANK trigger the recruitment of TRAF6 and stimulate the downstream MAPK and NF-κB signal pathways to initiate osteoclast formation [8,9]. Mitogen-activated protein kinase (MAPK) play a role by promoting the phosphorylation of related biomolecules downstream of the pathway, including ERK,JNK and p38 [10]. The activation of these factors can initiate the transcription of genes involved in osteoclast differentiation and bone resorption, including genes encoding tartrate-resistant acid phosphatase (*TRAP*), cathepsin K (*CTSK*), calcitonin receptor (*CTR*), and matrix metallopeptidase 9 (*MMP-9*), ultimately resulting in the formation of mature multinucleated osteoclasts [11,12].

Trifolirhizin is a natural plant active component isolated from the root of Sophora flavescens. Sun et al. and other studies have demonstrated that trifolirhizin intensifies the phosphorylation of AMPK in colorectal cancer cells, and prevents the phosphorylation of mTOR, leading to autophagy in colorectal cancer cell lines (HCT116 and SW620) [13]. In addition, it can inhibit the proliferation of human myeloid leukemia cells, hepatocellular carcinoma fine human ovarian cancer and lung cancer cells [14,15]. With the in-depth study of trifolirhizin bean sandaloside, it shows a strong research value. Currently, the research on the prevention, treatment of osteoporosis, and molecular mechanism of trifolirhizin remains limited. Our team obtained a variety of active components from plants and studied their effects related to bone metabolism and found that the trifolirhizin had a significant inhibitory effect on osteoclast differentiation.

Therefore, this study aims to determine the effects of trifolirhizin in regulating osteoclast differentiation and bone resorption. Furthermore, we also aim to evaluate its therapeutic potential for OVX-induced bone loss.

2. Materials and methods

2.1. Reagents

Trifolirhizin was purchased from Munster Company in Chengdu, China. Dimethyl sulfoxide (DMSO) and Fluoroshield with DAPI were purchased from American Sigma-Aldrich. Rat Source Recombinant M-CSF and Recombinant RANKL were purchased from R&D Systems in the United States. TRAP staining kit was purchased from LuShen Biotechnology in China. Anti-NFATc1 antibodies were purchased from Santa Cruz Biotechnology, United States. Anti-p-p38, p38, *p*-JNK, JNK, *p*-ERK, and ERK were purchased from CST Company, United States. Anti-β-actin and fluorescent secondary antibodies were purchased from PTG, Wuhan, China.

2.2. Mice

Wild type (WT) C57BL/6J mice, 8 weeks old, were procured from Beijing Weitong Lihua Company. The animals were raised in an SPF environment at the Animal Center of the Medical College of Shantou University. Mice were randomly divided into four groups: sham operation group, OVX group, OVX mice + low concentration treatment group, and OVX mice + high concentration treatment group. All groups were maintained under the same conditions, including photoperiod (12 h/d), temperature (20−26 centigrade), and humidity (40 %–70 %). Adequate water and feed were provided to all mice. According to the successful model of the research group, we established the OVX mouse model [11]. After 3 weeks of adaptive growth following purchase, ovariectomy was executed in all groups, except for the sham operation group, where the sham operation was carried out. Bilateral ovaries, including part of the fallopian tubes, were removed from the mice. After the operation, the mice were placed on a preheated constant temperature pad and injected with penicillin injection into the right hindlimb to prevent infection and improve the postoperative survival rate of mice. After a week of rest, the drug was given once a day by intraperitoneal injection, with each dose being 200 μl, and this treatment lasted for 6 weeks. The low concentration treatment group administered 10 mg/kg to OVX mice, while the high concentration treatment group administered 20 mg/kg. As a control group, the sham operation group and the control group received an injection of PBS enriched with 1 % DMSO. Following a 6-week treatment period, the mice were sacrificed, and their left lower limbs were excised and fixed with 4 % paraformaldehyde. Removal of any excess soft tissue enabled the preparation of the intact tibia for microcomputer tomography (μCT) analysis. The animal experiment was approved by the Ethics Committee of Medical College, Shantou University (NO.SUMC2022-207).

2.3. CCK-8 cell viability assay

Cell Counting Kit-8 (MCE, USA) was used to detect the effect of trifolirhizin on Bone marrow macrophage (BMM) activity according to the manufacturer's instructions. Bone marrow stromal cells were inoculated into 96-well plates at the density of 6000 cells per well, and cultured in complete α-MEM containing 10 % serum in 37 centigrade incubator overnight. Trifolirhizin was added to the medium and incubated for 48 h, then 10 μLCCK-8 reagent was added to each well. After 3 h, the absorbance (OD450) was measured at 450 nm wavelength by full-wavelength grating enzyme labeling instrument.(Tecan, Switzerland).

2.4. Osteoclastogenesis assay

Fresh BMMs were extracted from the bone marrow cavity of femur and tibia of C57BL/6J mice and cultured in T75 culture bottle with α-MEM medium containing 25 ng/mL M-CSF (hereinafter referred to as complete α-MEM). After about 3 days of culture, BMMs could proliferate to almost cover the culture flask, and then the cells were inoculated into 96-well plate at the concentration of 6000 cells per well (3 multiple holes) and cultured overnight. The cells were stimulated with 50 ng/mL RANKL, and different amounts of trifolirhizin were added according to the different groups. The culture medium was changed every other day until multinucleated osteoclasts were formed. The cells were fixed with 4 % paraformaldehyde. After 10 min, TRAP activity was detected through cell staining. Cells with more than 3 nuclei and positive TRAP were considered to be osteoclasts.

2.5. Bone resorption assay

The treated bone slices were placed in a 96-well plate, and the cells were inoculated on the surface of the bone slices at a density of 8000 cells per well. The cells were incubated overnight in a complete culture medium containing only M-CSF, then the medium was changed to a complete medium containing RANKL (50 ng/mL) and M-CSF, and different concentrations (0, 20, 40 μM) of trifolirhizin were added. In the control group, obvious osteoclasts and bone lacunae were observed. The culture medium was removed, and the cells were washed with PBS thrice. Next, 10 % sodium hypochlorite was added. Followed by another three washes with PBS. The bone resorption area was observed and imaged under a microscope. The bone resorption area was measured by Image J software.

2.6. RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

According to the guidelines provided by the manufacturer, Trizol was used to lyse the osteoclasts and subsequently extract the total RNA. The cDNA produced by RNA reverse transcription was quantified using real-time fluorescence quantitative PCR (qPCR). Every 20 μL reaction mixture contained 10 μL of Taq enzyme premix, 0.4 μl of forward and reverse primers and 2 μg of cDNA, which were supplemented to 20 μL with DEPC water. The PCR cycle conditions are as follows: temperature 95 centigrade, time 30 s as pre-denaturation step, temperature 95 centigrade for 10 s, temperature 60 centigrade for 30 s, 40 cycles as cycle reaction steps, melting curve is collected by default according to the instrument. The set of specific gene detection and quantitative primers is shown in Table 1. The reaction was carried out using ABIQS5RealTimePCR instrument (ABI, US). The expression of each target gene was calculated by using the 2-delta CT method. The CT value of each specific target gene was normalized with that of β-actin, and the ΔCT value was obtained. ΔΔCT can then be further normalized to the sample.

2.7. Western blot analysis

For short-acting proteins (JNK, p38, and ERK), cells were seeded in 6-well plates at a density of 8×10^5 cells/well and cultured in a complete medium containing only M-CSF in the incubator until the cells adhered. Subsequently, the medium in the 6-well plate was changed to a simple α-MEM medium for starvation culture. After 2 h, 100 μM trifolirhizin was added to each well in the treatment group, and 1/1000 DMSO was added to each well in the control group. The cells were returned to the incubator and further cultured for

Table 1
Primer sequences for qPCR.

Genes	Primer sequences (5′ → 3′)	
Mmp9	Forward	AAGGCAGCGTTAGCCAGAAG
	Reverse	GCGGTACAAGTATGCCTCTGC
Cathepsin K Ctsk	Forward	TAGCACCCTTAGTCTTCCGC
	Reverse	CTTGAACACCCACATCCTGC
Dc-stamp	Forward	ACCTAAGCGGAACTTAGACACA
	Reverse	AGGGCTTCGTGGAAACACAT
Acp5	Forward	TGTGGCCATCTTTATGCT
	Reverse	GTCATTTCTTTGGGGCTT
Nfatc1	Forward	CAACGCCCTGACCACCGATAG
	Reverse	GGCTGCCTTCCGTCTCATAGT
β-actin	Forward	CACTGTCGAGTCGCGTCC
	Reverse	TCATCCATGGCGAACTGGTG

2 h. In conclusion, the pre-diluted solution containing RANKL was introduced to attain a desired concentration of 50 ng/mL. Subsequently, samples were gathered at various time intervals (0, 5, 10, 20, 30, and 60 min). In the time-dependent experiment, the medium was changed at planned time points (1, 2, 3 days) to achieve a final concentration of 50 ng/mL RNAKL and 40 μM trifolirhizin. In the concentration-dependent experiment, a full medium fluid exchange was carried out on the first and third days after seeding. The concentrations of trifolirhizin sandaloside used were 0, 10, 20, and 40 μM, and samples were collected when mature osteoclasts appeared.

During sample collection, 200 μL RIPA lysate (Beyotime) was added to each Petri dish, and total protein was extracted and quantified. Phosphatase inhibitors were added for the extraction of phosphorylated proteins. 20ug proteins were added to each lane for SDS-PAGE gel electrophoresis, and then the protein bands were transferred to NC membrane (Pall, USA). The membrane was then sealed with 5 % skim milk at room temperature for 1 h. After TBST cleaning, the primary antibody was added and incubated overnight at 4 centigrade. After another round of TBST cleaning, the secondary antibody was added and incubated at room temperature for 1 h. After cleaning, the antibody reaction was detected using a gel imaging system (Bio-Rad), Image J software was used to quantify the grayscale values.

2.8. Micro-computed tomography (μCT) and histological assessments

After the tibia was removed from the mice and pretreated, the washed tibia was loaded into a 1.5 ml EP tube filled with PBS to maintain position and moisture. Then fix the test tube to the specimen shaft according to the instrument instructions. Each tibia was scanned using the Skyscan1176 μm CT system (Bruker microCT, Kontich, Belgium). A total of 50 pieces under the growth plate with a height of 100 pieces were selected as the observation area (ROI). CTAn was employed to measure the cancellous bone parameters within the ROI. The other tibia's tissue was decalcified, embedded in paraffin, and subsequently sectioned to obtain 5 mm thick slices.

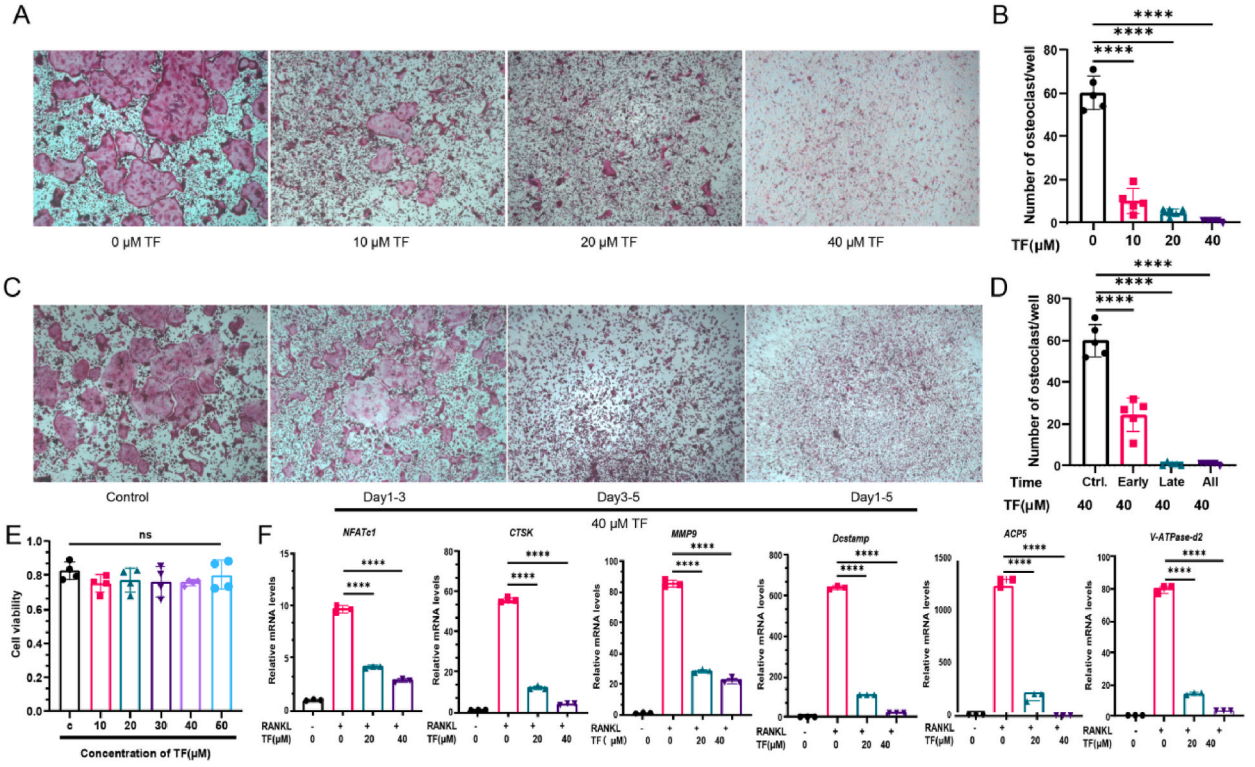

Fig. 1. Trifolirhizin inhibits RANKL-induced osteoclast differentiation and gene expression. (A) Representative images of osteoclast culture treated with varying
doses of TF for 5 days. We repeated the experiment three times. (B) Quantification of TRAP positive multinucleated cells following treatment with varying doses of TF. The data represent as mean ± SD. $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to trifolirhizin-untreated controls. (C) Representative images of osteoclasts cultured after 40uM TF were given at different times. We repeated the experiment three times. (D) Quantification of TRAP positive multinucleated cells after administration of 40uM TF at different times. (E) Proliferation of M-CSF stimulated BMMs following incubation with TF at different doses for 48 h as measured by CCK-8 assay. We repeated the experiment three times. (F) Real-time PCR analyses was performed on RNA extracted from cells stimulated for 5 days with RANKL and varying doses of TF. Gene expression of osteoclast marker genes *NFATc1, CTSK, MMP9, DC-STAMP, ACP5 and V-ATPase-D2* was normalized to β-actin RNA and then compared to RANKL-only control samples to obtain the relative fold change. We repeated the experiment three times. The data represent as mean ± SD. $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to trifolirhizin-untreated controls.

These sections were stained using hematoxylin and eosin, and their TRAP activity was evaluated. The sections were observed using an Observer A1 phase contrast inverted fluorescence microscope.

2.9. Statistical analysis

All data are expressed as mean ± standard deviation. Independent *t*-test was used to compare two independent samples. One-way ANOVA was used to compare the single-factor samples among multiple groups. The difference was statistically significant for $P < 0.05$.

3. Result

3.1. Trifolirhizin inhibits RANKL-induced osteoclast differentiation and gene expression

The biological effect of trifolirhizin on osteoclast formation was studied using osteoclast differentiation experiments *in vitro*. The results showed that trifolirhizin could significantly inhibit the formation of multinucleated osteoclasts induced by RANKL in a concentration-dependent manner. (Fig. 1A and B). To understand the effect of trifolirhizin in different stages of osteoclast differentiation, we added trifolirhizin in the pre-and post-stage of osteoclast differentiation (Prophase: 1–3 days of induction; later: 3–5 days of induction). The results showed that trifolirhizin inhibited osteoclast differentiation in both stages, and the effect on the latter stage was more obvious (Fig. 1C and D). The cytotoxic effect of trifolirhizin sandalwood glycoside on BMMS was detected through the CCK-8 method. According to the findings, trifolirhizin displayed effective inhibition of osteoclast formation within the dosage range of 10–40 μM. In addition, after 48 h of culture, the results showed that it had no cytotoxicity to bone marrow-derived macrophages. (Fig. 1E). The inhibition of osteoclast formation was further confirmed through the verification of trifolirhizin's impact on the expression of genes specific to osteoclasts, as determined by real-time quantitative polymerase chain reaction. Trifolirhizin inhibited osteoclast marker gene expression in a dose-dependent manner, including *NFATc1, CTSK, MMP9, DC-STAMP, ACP5, and V-ATPase-D2* (Fig. 1F).

3.2. Trifolirhizin inhibits osteoclast fusion and bone resorption induced by RANKL

The fusion of osteoclast precursors is necessary conditions for the production of giant multinucleated osteoclasts and subsequent bone resorption. Our bone resorption experiment showed that trifolirhizin could significantly reduce the bone resorption area, which was confirmed by the inhibition of osteoclast formation (Fig. 1A and B) and the reduction of osteoclast fusion factor *DC-STAMP* expression. (Fig. 1D). When cultured on bone or osteoid matrix (such as hydroxyapatite), pseudopod corpuscles rapidly reorganized into a compact structure called F-actin ring, which is essential for bone resorption and sealing the underlying cells. As shown in Fig. 2A, after cultured on bovine bone slices for 48 h, the osteoclasts in the blank group showed extensive bone resorption characteristics, while cells treated with 20 and 40 μM trifolirhizin showed significant bone resorption disorders. The absorption area decreased significantly after 20 and 40 μM trifolirhizin treatment (Fig. 2B). The findings suggest that trifolirhizin has the potential to not only impede osteoclasts' formation but also hinder their bone resorption capabilities. However, the specific mechanism of trifolirhizin's inhibitory effect on bone resorption of osteoclasts requires further investigation.

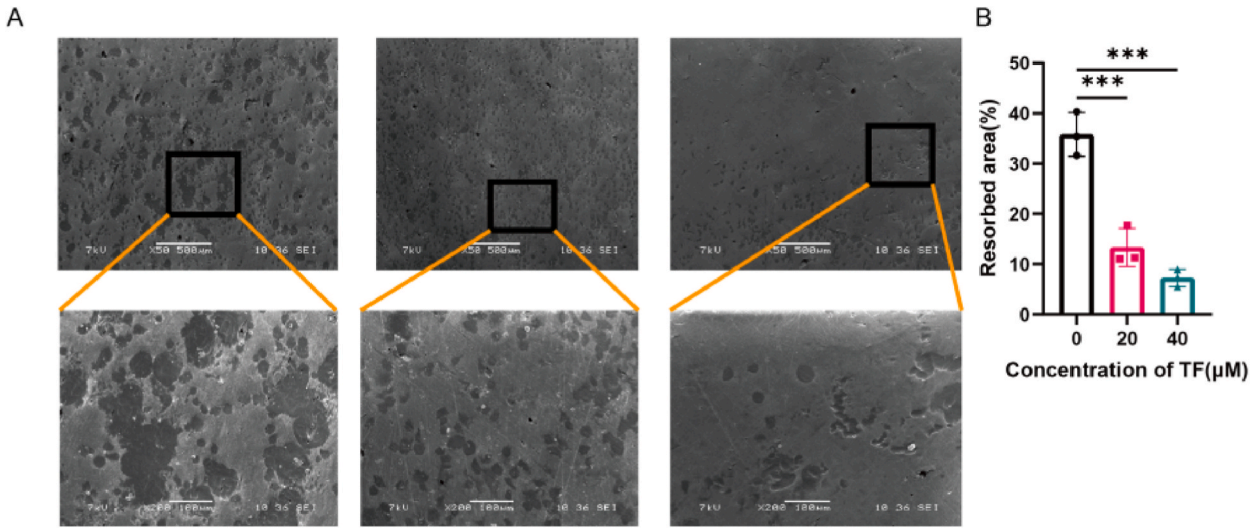

Fig. 2. Trifolirhizin inhibits osteoclast fusion and bone resorption induced by RANKL. (A) Representative images of bone resorption lacunae formed after treatment with different concentrations of TF. (B) Percentage of the area of bovine bone slices surface resorption.The data represent as mean ± SD. $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to trifolirhizin-untreated controls.

3.3. Trifolirhizin inhibits RANKL-induced MAPK signal transduction

MAPK signal pathway is an important pathway in osteoclast differentiation. Western blotting was used to detect the changes in the MAPK signal transduction pathway to explore the possible mechanism through which trifolirhizin inhibits osteoclast formation induced by RANKL. As shown in Fig. 3A and B, 40 μM trifolirhizin could inhibit RANKL-induced JNK phosphorylation and ERK phosphorylation. However, the phosphorylation of p38 was inhibited only in the early stage (5 min), but not at other time points.

3.4. Trifolirhizin inhibits RANKL-induced NFATc1 expression

NFATc1 plays a crucial role as a transcription factor in the differentiation of osteoclasts. The primary regulatory pathways that govern the expression of NFATc1 are the RANKL-induced MAPK and NF-kB signaling pathways. We studied the effect of trifolirhizin on the expression of NFATc1 protein using Western blotting analysis. The results revealed that the expression of NFATc1 protein was inhibited by trifolirhizin, and the inhibitory effect increased with the increase of concentration(Fig. 3C and D). In addition, osteoclasts were induced by RANKL at different time points (days 1, 2, and 3), and trifolirhizin was added during the whole process. The results showed that trifolirhizin could inhibit the expression of NFATc1 proteins at different time points of osteoclast differentiation(Fig. 3E and F).

In summary, our *in vitro* data show that trifolirhizin shows an anti-osteoclast effect by weakening RANKL-induced MAPK-NFATc1 signaling pathways.

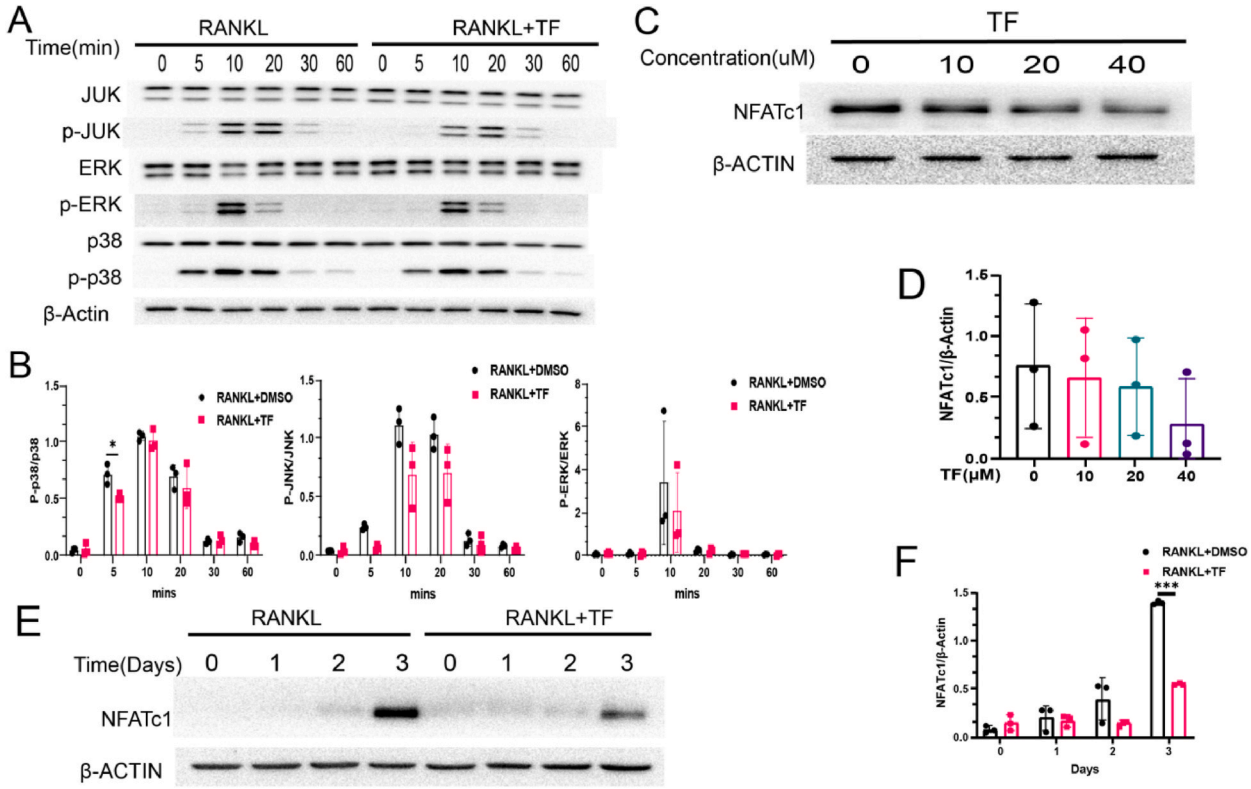

Fig. 3. Trifolirhizin inhibits RANKL-induced MAPK signal transduction and NFATc1 expression. (A) BMMs pretreated with TF(100 μM) for 2h were then stimulated with RANKL (100 ng/mL) for 0, 5, 10, 20, 30, and 60 min. Protein was then extracted for Western blot analyses using antibodies as shown. We repeated the experiment three times. (B) The ratios of the density of p-p38 bands relative to total p38 band, *p*-JNK bands relative to total JNK bands, *p*-ERK bands relative to total ERK were then determined using Image J. The data represent as mean ± SD. $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (C) Western blot was performed on protein extracted from cells stimulated for 5 days with RANKL and varying doses of TF. We repeated the experiment three times. (D) The ratios of the density of NFATc1 bands relative to β-actin were determined using Image J. The data represent as mean ± SD. $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (E) BMMs were stimulated with RANKL (50 ng/mL) for 0,1,2 and 3 days and treated with TF (40 μM) throughout the whole process. Protein was then extracted for Western blot analyses using antibodies against NFATc1 and β-actin antibodies. We repeated the experiment three times. (F)The ratio of the density of NFATc1 bands relative to β-actin bands shown below was determined using Image J. The data represent as mean ± SD. $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3.5. Trifolirhizin inhibits OVX-induced bone loss

To further clarify the therapeutic effect of trifolirhizin on diseases related to bone resorption, we established an ovariectomized (OVX) model. The mice were divided into two groups: sham operation group and OVX group, and OVX group included control group and administration group. The mice in the treatment group were injected intraperitoneally with 10 mg/kg (low dose) and 20 mg/kg (high dose) respectively. Sham operation group and OVX control group were injected with PBS, once a day for 6 weeks. μCT scanning of the extracted tibia showed extensive loss of trabeculae in ovariectomized mice(Fig. 4A). The analysis of trabecular related parameters of μCT data showed that the bone loss caused by ovariectomy was significantly reversed in the high dose group, but there was no significant change in the low dose group(Fig. 4A,B). The histological assessment revealed a notable reduction in the quantity of TRAP-positive osteoclasts within the trifolirhizin treated group compared to the ovariectomized group. The latter showed a large number of TRAP-positive osteoclasts and obvious trabecular reduction and bone loss (Fig. 4C). In conclusion, our results propose that the bone loss induced by OVX can potentially be mitigated through the suppression of osteoclast generation *in vivo* using trifolirhizin.

According to our experimental results *in vivo* and *in vitro*, it is proved that trifolirhizin can hinder the expression of related genes in the process of osteoclast differentiation and maturation. This inhibition is achieved by targeting the phosphorylation of factors related to the MAPK-NFATc1 signal pathway. Finally, the result of this molecular action leads to the inhibition of osteoclast formation and the decrease of bone resorption activity(Fig. 5).

4. Discussion

Osteoporosis, a prevalent disorder marked by reduced bone density and deterioration of microarchitecture, leads to bone fragility and escalates the likelihood of fractures [16]. The incidence of this condition increases with age, starting from 6 % at 50 years and reaching 50 % at 80 years [17]. The incidence of osteoporosis-related fractures is higher in women, with data showing an increase in the incidence of fractures in about 50 % of women and 20 % of men over the age of 50. Osteoporosis has significant consequences, including long-term disability, reduced quality of life, and increased mortality rates [18,19]. This condition is a prevalent health issue

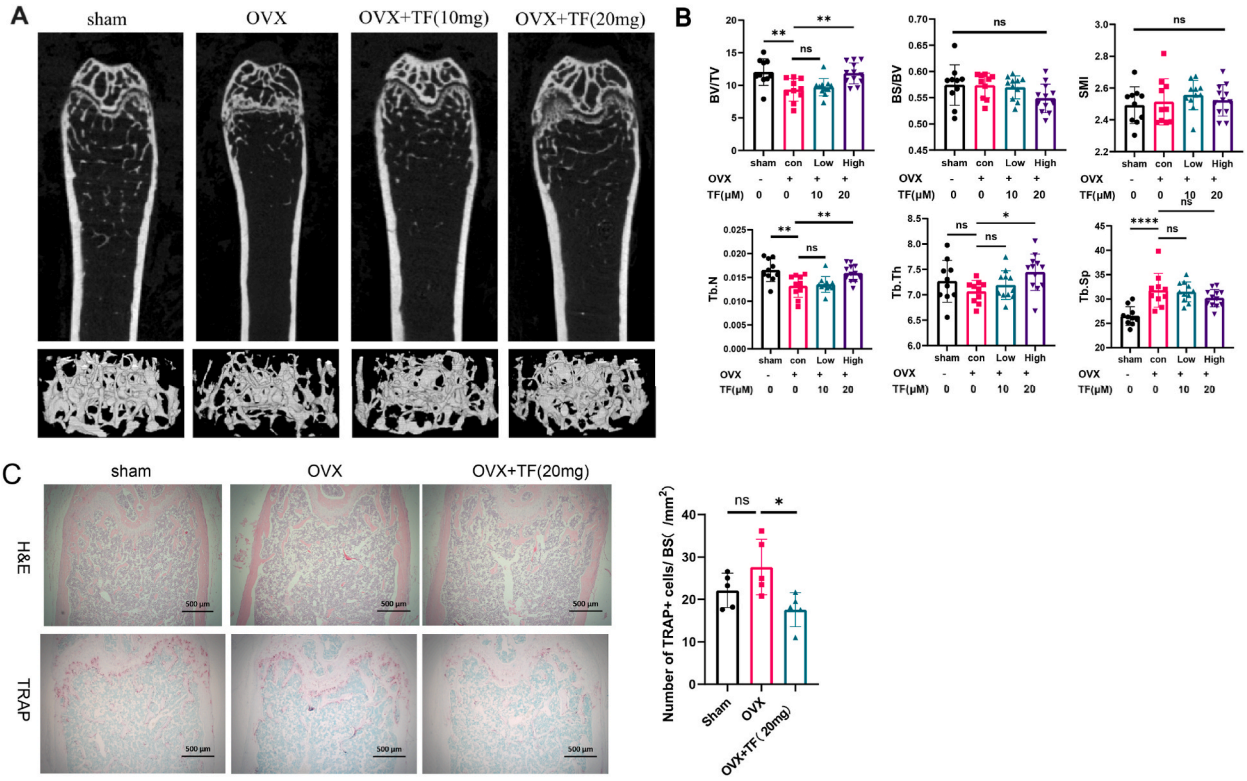

Fig. 4. Trifolirhizin inhibits OVX-induced bone loss. (A) Representative 3D reconstructions of trabecular bone from the tibia of sham mice, OVX mice, OVX mice treated with 10 mg/kg TF and OVX mice treated with 20 mg/kg TF, showing the protective effect of TF treatment following OVX. (B) Quantitative analyses of bone volume/tissue volume (BV/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular thickness (Tb.Th) and bone mineral density (BMD), bone surface/bone volume (BS/BV) and structure model index (SMI). The data represent as mean ± SD. $^{ns}p>0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ relative to OVX control. (C) Representative images of decalcified bone stained with H&E and TRAP and number of TRAP-positive osteoclasts regarding TRAP-stained bone sections (n = 5) from sham mice, OVX mice and OVX mice treated with TF 10 mg/kg. The data represent as mean ± SD. $^{ns}p>0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

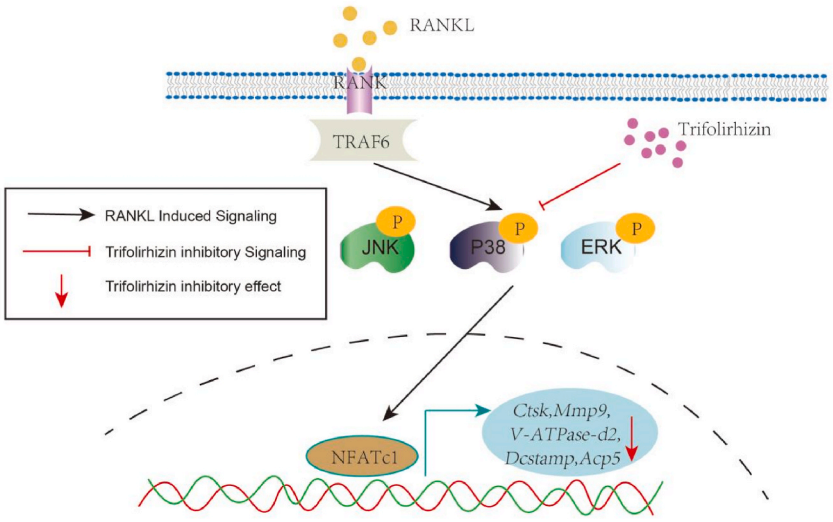

Fig. 5. The diagram of molecular regulation mechanism.
Trifolirhizin can inhibit MAPK-NFATc1 pathway, and then inhibit the expression of osteoclast differentiation marker genes, and finally inhibit osteoclast production and bone resorption activity *in vivo* and *in vitro*.

in China's aging society and has also become prevalent in developed and developing countries, resulting in substantial social and economic burdens. While the pathophysiological mechanism of osteoporosis is well understood, there is currently no definitive clinical treatment for this condition. Therefore, it is imperative to investigate effective medications for the prevention and treatment of osteoporosis.

Trifolirhizin, derived from the root of Sophora flavescens, is a natural compound found in rosewood. At present, the research on trifolirhizin mainly focuses on anti-tumor and anti-inflammatory effects [13,15,20], the role and mechanism in bone has not been explored to a large extent. Our results show that trifolirhizin can effectively inhibit osteoclast differentiation and maturation and bone resorption efficiency, and the higher the concentration, the greater the effect. Notably, the addition of trifolirhizin demonstrates pronounced inhibitory effects in both early and late stages of differentiation, with greater efficacy observed in the latter. Furthermore, we meticulously investigated the underlying regulatory pathway of trifolirhizin.

In postmenopausal women, osteoporosis results from the increase of osteoclasts and the enhancement of bone resorption [15]. Osteoclasts differentiate from hematopoietic stem cells, and their transformation into fully activated multinucleated osteoclasts relies on the existence of RANKL and the allowable function of M-CSF. After being stimulated by RANKL, the activities of several regulatory transcription factors and enzymes of osteoclasts are enhanced to promote the differentiation and proliferation of osteoclasts, thus enhancing bone resorption [21,22]. RANKL-RANK binding rapidly triggers downstream signaling pathways, in which NF-κB and MAPK cascades play a key role in the initial stage of monocyte progenitor cells participating in osteoclast lineage [23]. Downstream regulators associated with mitogen activated protein kinase (MAPK), such as ERK, JNK and p38, are further activated [24].The activation of these factors further promotes the transcription of genes related to osteoclast differentiation, including genes encoding TRAP, CTSK, CTR and MMP9, which eventually lead to osteoclast maturation [10,12].

In this study, trifolirhizin could inhibit the phosphorylation level of MAPK-activated protein pathways. Among them, the most obvious inhibitory effect was observed on JNK protein, with phosphorylation levels in the trifolirhizin treatment group at 5, 10, and 20 min decreased significantly. Additionally, the inhibitory effect of trifolirhizin on p38 phosphorylation only occurred in the early stage of differentiation, but had no significant effect in the middle and late stages.

Surprisingly, the phosphorylation level of ERK reached its peak at 10 min, and the treatment group with trifolirhizin had a significant inhibitory effect at this time point. However, the total protein expression of ERK in the trifolirhizin treatment group decreased significantly at the corresponding time point when the phosphorylation level increased. The reason for this phenomenon may be that in the relevant cell research work, in order to better carry out related cell experiments, the low serum method is often used to synchronize the cell cycle, that is, to obtain a large number of synchronous cells and make the cell cycle close to G0 or G1 phase. In this study, BMMs cells were treated with low serum for 4 h before validating the short-acting protein (2 h before and 2 h after the addition of trifolirhizin). However, prolonged low-serum culture time led to a decrease in ERK total protein expression at the corresponding time point when the phosphorylation level increased. This inevitably affected the protein expression level to some extent. Therefore, in the follow-up experiments, it is suggested to shorten the low-serum culture time during cell culture to reduce the impact on the target protein.

NFATc1, also referred to as NFAT2 or NFATc [10], belongs to a transcription factor gene family known as NFAT (activated T nuclear factor). RANKL stimulation induces strong expression of NFATc1, making it the most prominent transcription factor gene. A key player in osteoclast differentiation and formation, NFATc1's activation is implicated in numerous signal pathways associated with osteoclast formation [25,26]. Research has demonstrated that activated MAPK can trigger the translocation of NFATc1 to the nucleus

via phosphorylation, leading to increased expression of CTSK and TRAP [27]. Within this investigation, it has been preliminarily verified the inhibition of trifolirhizin on the MAPK pathway. Furthermore, the expression of NFATc1 protein was significantly inhibited after treatment with trifolirhizin, which was positively correlated with time and concentration. However, further proof is lacking. If reverse verification can be performed through molecular docking or the addition of corresponding protein agonists, the conclusion will be more convincing. We will also conduct supplementary research in future studies.

In addition to its ability to hinder the formation of osteoclast progenitor cells, our research also uncovered the inhibitory impact of trifolirhizin on the process of bone breakdown conducted by osteoclasts. When osteoclasts were treated with trifolirhizin and cultured on slices of bovine bone, their ability to resorb bone was notably diminished. Furthermore, our *in vivo* study utilizing mice with an OVX model demonstrated that trifolirhizin possessed the potential to safeguard against the detrimental effects of bone loss. Our study lacked an active treatment control group (such as estrogen therapy or bisphosphonate therapy), but the high-concentration group displayed a nearly complete reversal of the bone loss resulting from OVX, showcasing the remarkable protective qualities of trifolirhizin. Concurrently, examination of the histological samples revealed a substantial reduction in the presence of TRAP positive osteoclasts on the bone surface, thereby minimizing the extent of bone loss triggered by ovariectomy.

This investigation elucidated that trifolirhizin possesses the capability to efficaciously impede the generation of osteoclasts and hamper their activity in resorbing bone. Regrettably, our inquiry has not delved deeply into the realm of osteogenesis. Nevertheless, the outcomes of this examination furnish substantiation supporting trifolirhizin's potential as a pharmaceutical agent in the management and prophylaxis of osteoporosis and other conditions characterized by bone loss.

Ethics statement

The animal experiment was approved by the Ethics Committee of Medical College, Shantou University (NO.SUMC2022-207).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Zihong Lin: Writing – original draft, Methodology, Data curation, Conceptualization. **Zhigao Zhou:** Writing – review & editing, Validation, Investigation. **Jiajie Ye:** Supervision, Formal analysis. **Jinfu Wei:** Validation, Resources. **Shaozhe Chen:** Supervision, Software. **Wenyun Zhou:** Investigation, Formal analysis, Visualization, Formal analysis. **Yonghao Bi:** Software, Formal analysis. **Zibin Zhou:** Software, Formal analysis. **Gang Xie:** Software, Formal analysis. **Guixin Yuan:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Guanfeng Yao:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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