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Ethanol extract of Cyathulae Radix inhibits osteoclast differentiation and bone loss

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[ABSTRACT] Objective: Cyathulae Radix is a traditional Chinese medicine and a traditional vegetable in China for several thousand years. It can enhance bone density, promote metabolism, and effectively alleviate the pain caused by osteoporosis. However, the underlying molecular mechanisms of Cyathulae Radix on osteoporosis have not been disclosed yet. Methods: To investigate the effect and mechanism of Cyathulae Radix ethanol extract (CEE) on inhibiting osteoporosis and osteoclastogenesis, 8-week-old female mice were ovariectomized and treated with CEE for 8 weeks. Micro-computed tomography was used to evaluate the histomorphometric parameters. Bone resorption activity was determined by TRAP staining. qRT-PCR were used to detect the expression levels of TRAP, RANK, and OPG in serum. Western blot and qRT-PCR were used to detect the expression of osteoclastogenesis-related markers. Results: Compared with only ovariectomy (OVX) group, CEE could increase the density of bone trabecula and the maximum load pressure of tibia. CEE up-regulated BV/TV and Tb.Th, then down-regulated BS/BV and SMI. E2 and OPG levels in serum were significantly up-regulated in CEE treatment group, while RANK was down-regulated. The gene and protein levels of MMP-9, Cathepsin K and TRAP were down-regulated by CEE. In the process of BMMs differentiating into osteoclasts, CEE significantly decreased the number of mature osteoclasts, the bone resorption pit areas and the expression levels of MMP-9, Cathepsin K and TRAP on gene and protein levels. Compared with DMSO group, CEE significantly inhibited the expressions of RANK, TRAF6, c-Fos and NFATc1 in osteoclast. And the phosphorylation levels of ERK, JNK and p65 were reduced by CEE during osteoclastogenesis. Conclusion: CEE can significantly inhibit OVX-induced osteoporosis in mice and RANKL-induced osteoclastogenesis in BMMs. It may be related to the inhibition of expression of RANK, TRAF6, c-Fos, NFATc1 and the phosphorylation of ERK, JNK and p65 in ER/RANK/NFATc1 signaling pathway.

[KEY WORDS] Osteoporosis; Osteoclast; BMMs; Cyathulae Radix; RANKL; ER/RANK/NFATc1 Signaling Pathway

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Introduction

Osteoporosis is a kind of systemic osteopathy, which is characterized by the bone microstructure destruction, the decrease of bone mass, quality and strength, and the patients are prone to fracture. The bone integrity is maintained by the dynamic equilibrium of bone absorption and formation. Osteoclasts play an important role in bone resorption. Active osteoclasts secrete acidic materials and enzymes which can decompose bone minerals, resulting in bone loss. When osteoclasts are more active than osteoblasts, bone resorption is greater than bone formation. Then osteoporosis occurs. In the 5-10
years after menopause, the endocrine function of ovary is disorder or declining, which leads to the estrogen level decrease. The bone resorption ability of osteoclasts was increased and the bone resorption is excessive, which leads to postmenopausal osteoporosis [1,2].

Osteoclast related indexes are commonly used in osteoporosis diagnosis and treatment. Osteoclast differentiation, maturation and ability are regulated by various factors. This process involves complex and intertwined signal pathways. RANKL is necessary for osteoclast differentiation. OPG-RANKL-RANK signaling pathway is an important intercellular signal transduction pathway in osteoclast formation and differentiation [3]. M-CSF can promote osteoclast differentiation and promote the combination of RANK and RANKL. RANK recruits TRAF6 followed by downstream pathways activation including NF-κB, MAPK and c-src-P3K-Akt signaling pathways [24]. NF-κB is an important regulator of the survival and function of mature osteoclasts. NF-κB promotes osteoclast differentiation and prevents apoptosis after entering the nucleus [9]. When in resting state, NF-κB tightly binds to the inhibitor protein IκB in the form of p50/p65. After up-regulation of IκBα early expression, the negative feedback loop is started, thus the intranuclear transfer of p65/p50 is limited [10]. MAPK signaling pathway includes ERK, JNK and p38 pathways. Activation of ERK, JNK and p38 MAPK can increase the expression of NFATc1 and promote differentiation and function of mature osteoclasts [11-14]. Blocking p38 pathway can inhibit osteoclast differentiation, maturation and decrease local bone resorption [15]. GSK3β prevents NFATc1 from binding to DNA [16] and Akt activation can block GSK3β [17]. Activation of these pathways transmits signal to c-Fos and NFATc1 to affect osteoclast formation [18,19]. NFATc1 plays an important role in transcriptional regulation of osteoclasts. After NFATc1 activation, it was transferred from cytoplasm to nucleus, and osteoclast specific genes such as TRAP, CTSK and MMP-9 were transcribed [20]. ERα is an essential molecule for anti-osteoporosis role of estrogen. It can inhibit osteoclast differentiation by decreasing RANK [21].

Cyathulae Radix has been listed in the Pharmacopoeia of the People’s Republic of China. As the dry root of Cyathula officinalis Kuan, Cyathulae Radix is a common traditional Chinese medicine used to treat various orthopedic diseases, such as bone injury, osteoarthritis and rheumatic arthritis [22]. It is also a traditional vegetable in China. As a kind of dietary supplement approved by National Health Commission of the People’s Republic of China, Cyathulae Radix has extensive pharmacological effects, including effects on growth and articulation, anti-inflammatory effect, antioxidation effect, antihypertensive effect and antitumor effect [23]. Wang et al. [24] revealed that Cyathulae Radix has a weak estrogen-like effect in ovariectomized rats. It may be related to the treatment of osteoporosis. Additionally, it had revealed osteo-protection in ovariectomized rats [25]. The main components of Cyathulae Radix are triterpenoid saponins, steroid ketones, polysaccharides, etc. Steroid ketones have been proved to have anti-osteoporosis effects, including ecdysterone, cystinone, etc. [26,27]. Estrogen deficiency is the main cause of postmenopausal osteoporosis. And cyasterone was found as an authentic standard in the Cyathulae Radix extract that not only has a significant estrogen-like effect, but also has a significant bidirectional effect of inhibiting osteoclastic differentiation and promoting osteogenic differentiation [28]. Triterpenoid saponins and polysaccharides of Cyathulae Radix have anti-inflammatory activity. Triterpenoid saponins mainly include cyanoside A, cyanoside B, etc. [29]. Polysaccharide can down-regulate the proportion of CD4+CD25 +Foxp3 + Tregs cells [30]. However, the underlying molecular mechanisms of Cyathulae Radix on osteoporosis have not been disclosed yet.

Therefore, the objective of this study was to explore the preventive and therapeutic effects of Cyathulae Radix ethanol extract in vitro and in vivo. It will benefit significantly the development and utilization of Cyathulae Radix.

Materials and Methods

Preparation of Cyathulae Radix ethanol extract and estradiol

Cyathulae Radix was purchased from Hengyue Herbal Pieces Co., Ltd. (Hunan, China, Lot: 17101304). 30-fold ethanol (70% v/v) was added and Cyathulae Radix was extracted at 85 °C for two times, 2 h each time. Extracted solution was concentrated. Polysaccharide was removed, then ethanol was volatilized to dryness. Water was added in Cyathulae Radix ethanol extract (CEE) to dissolve and dilute. 4.76 mg/mL CEE (Calculated by Cyathulae Radix concentration) was high dose (HD) and 1.19 mg/mL CEE was low dose (LD) for mice. DMSO was added in CEE to dissolve and dilute for cell culture.

1 slice (1 mg) of estradiol valerate tablet (Bayer China, Lot: 395A) was ground into powder. Water was added to dilute estradiol (E2) to 0.03 mg/mL for mice.

High-performance liquid chromatography analysis

Ethanol extract from Cyathulae Radix was filtered through syringe filter Millipore (0.45 μm, 50 mm, PES). All compounds in the extract were determined using the LC-2010AHT HPLC System (Shimadzu, Kyoto, Japan). HPLC analyses were performed on a reverse phase Gensial® C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was 0.2% acetic acid in water (A) and methanol (B) mix using a linear gradient program: 0 min to 10 min of 70% A, decreasing the ratio to 55% A from 10 min to 40 min, and finally reaching 5% A from 40 min to 50 min. The flow rate was 1 mL/min and the injection volume was 10 μL. The column temperature was maintained at 35 °C, and detection was carried out at a wavelength of 243 nm (Figure 1).

For determination of Cyasterone in CEE, Cyasterone (2.03 mg) was exactly weighed and dissolved in 10 mL methanol to give serial concentrations. The calibration curve was
constructed by plotting the peak-area ratio of Cyasterone versus molar ratio of Cyasterone. The concentration of Cyasterone in CEE was over 1.05%, calculated by the following formula: $Y = 9264.2X + 11216, R^2 = 0.9998; 5.075-81.200 \mu g/mL.$

**Cell cultures and toxicity assessment**

Bone marrow macrophages (BMMs) were extracted from femora and tibias of 5-week-old female C57BL/6 mice. Bone marrow cavity was flushed with α-MEM to harvest bone marrow cells. Cells were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Solarbio, USA). At 37 °C in 5% CO$_2$, the non-adherent cells were collected after 12 h. Then cells were cultured with M-CSF (30 ng/mL) for 4 d. BMMs were treated with RANKL (50 ng/mL) and M-CSF (30 ng/mL) and induced for 4-6 d to form osteoclasts. Different concentrations of CEE and 5 μM E$_2$ were added to inter-

vene osteoclast induction respectively.

CCK-8 reagent was used to assess the cytotoxicity of CEE on BMMs. After BMMs were seeded in 96-well plates for 24 h, different concentrations of CEE (0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.125 mg/mL, 0.15 mg/mL, 0.3 mg/mL) were added. 48 h later, CCK-8 reagent was added to culture medium and incubated 3 h at 37 °C in 5% CO$_2$. The optical density was measured at a wavelength of 450 nm by microplate reader.

**TRAP staining**

BMMs were treated with RANKL (50 ng/mL) and M-CSF (30 ng/mL). After induced for 6 d to form osteoclasts, BMMs were washed with PBS buffer and fixed in 4% paraformaldehyde. Cells were stained using a TRAP staining kit according to the manufacturer’s instructions. The TRAP-positive cells containing three or more nuclei were considered as

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**Fig. 1** High-performance liquid chromatography analysis. (a) HPLC chromatogram of CEE. (b) HPLC chromatogram and chemical structure of Cyasterone. The peak of Cyasterone in CEE was identified by retention time (min.) in comparison with that in HPLC chromatogram of standard Cyasterone. The retention time of Cyasterone from CEE was 30.842 minutes.
osteoclasts, followed by counting osteoclasts under the inverted microscope (NIS-Elements F 4.0).

**Pit formation assay**

BMMs stimulated by M-CSF and RANKL were cultured on 100-200 μm thick bone slices in the presence or absence of CEE. After 8 d culture and pit formation, the bone slices were washed with PBS and fixed in 4% paraformaldehyde for 20 min. The bone slices were stained with toluidine blue dye (Sloarbio, China) for 10 min. Pit areas on slides were photographed with an inverted microscope and measured using Image-pro-plus 6.0 software.

**Animals and experimental treatments**

In total, 60 female ICR mice were purchased from Hunan SJA Laboratory Animal Co. Ltd. They were housed and acclimated in specific pathogen-free (SPF) conditions at the research animal laboratory. 8-week-old female mice were assigned to 6 groups and given oral gavage (n = 10/group): Blank, SHAM-operated (SHAM), OVX-mice (OVX), OVX-mice treated with 0.03 mg/mL E₂ (OVX-E₂), OVX-mice treated with 1.19 mg/mL CEE (OVX-LD), OVX-mice treated with 4.76 mg/mL CEE (OVX-HD). The Blank group mice were done without any treatment. The SHAM group mice were removed fat around ovaries. Mice of other groups were removed bilateral ovaries. At 8 weeks post-surgery and treatment, all mice were sacrificed and obtained the serum, tibiae and femora for analysis.

**Micro-computed tomography (Micro-CT) analysis**

The left femur of mice was taken and bone trabecula in the distal femoral metaphysis was measured by Micro-CT (NEMO® Micro-CT, PINGSENG Healthcare (Kunshan) Inc., China). Scanning regions were confined to the distal femoral metaphysis, extending proximally 1 mm from the growth plate, all mice were sacrificed and obtained the serum, tibiae and femora for analysis.

**Histology**

After sampling, femora were harvested and fixed in 4% paraformaldehyde. After decalcified with 10% EDTA, bones were embedded in paraffin, then cut into longitudinally 5-μm-thick sections. Hematoxylin and eosin (H&E) staining was used to observe histomorphology of the distal femur. Section images were acquired using M565 J/E/G Fluorescence inverted microscope (Nikon, Japan) [33, 34].

**Mechanical properties testing**

Three-point bending test was used to detect the mechanical properties of tibias. Bones were positioned on the biomechanics tester with a span width of 2 cm (MTS Insight 30, MTS Industrial Systems Co., Ltd, USA). Tibias were transversely pressurized at midpoint with speed of 1 mm/min until broken. Load and displacement data were collected from which ultimate force (N) were obtained.

**ELISA assay**

The blood sample was obtained and centrifugated to get serum. The levels of E₂, RANKL and OPG in serum were measured by using ELISA assay (BangYi, Shanghai, China). Operational process was according to the instruction.

**Quantitative RT-PCR**

The femora were frozen in liquid nitrogen and ground into powder to isolate RNA. Total RNA of femora and osteoclasts was extracted using TRizol reagent (Invitrogen) following manufacturer’s protocol. Reverse transcription PCR was performed with 1.0 μg total RNA and cDNA was synthesized (Thermo, USA). qRT-PCR was performed using specific primers (Table 1).

**Western blot**

Protein of femora was extracted by TRizol following manufacturer’s protocol. Cells were lysed with RIPA Lysis buffer. The protein concentration was determined by BCA method. Protein samples (30 μg) were separated in SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in blocking buffer (5% BSA or 5% skim milk) for 2 h, then probed with primary antibodies (TRAP, Cathepsin K (novus, USA), MMP-9 (R&D, USA), RANK, ERα, c-Fos, NFATc1, p-Akt, p-p65, p-ERK, p-JNK, p-p38, p-IκBα, Akt, p65, ERK, JNK, p38, IkBα (CST, USA), GAPDH (proteintech, USA), TRAF6 (abcam, USA)) against specific antigens at 4 °C overnight. Then the PVDF membranes were washed with PBS and fixed in 4% paraformaldehyde. After incubation with 1% skim milk (Thermo, USA) at room temperature for 1 h, the membranes were incubated with primary antibodies overnight. After washing, the membranes were incubated with secondary antibodies for 1 h at room temperature. The bands were visualized using enhanced chemiluminescence (ECL) Western Blotting Detection Reagents (Thermo, USA).

**Table 1 Primer sequences for qRT-PCR analysis of gene expression.**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctsk</td>
<td>5'-GCTTGGCATCTTCTCCAGTTTTA-3'</td>
<td>5'-GACACTGCATATTACGTACAA-3'</td>
</tr>
<tr>
<td>Mmp-9</td>
<td>5'-CAAAGACCTGAAAACCTCCACAC-3'</td>
<td>5'-GACTGCTTCTCTCCCCATCATC-3'</td>
</tr>
<tr>
<td>Trap</td>
<td>5'-CAGAAGACCTGCGACATTGTTA-3'</td>
<td>5'-ATCCATAGTGAACCCCGGAAT-3'</td>
</tr>
<tr>
<td>Era</td>
<td>5'-CTACTACCTGGAGAAGCAGC-3'</td>
<td>5'-GCTGCGATTCGACTCAGTAATAG-3'</td>
</tr>
<tr>
<td>Rank</td>
<td>5'-GAAGATGCTTTCTGGTGTTG-3'</td>
<td>5'-GCGTCATGTTGATCCGAT-3'</td>
</tr>
<tr>
<td>Nfatc1</td>
<td>5'-GGCTGCTCTTCGATCCTACATC-3'</td>
<td>5'-GCTGCTCTGCTGTTGCTTCCC-3'</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5'-CTCATGACCACAGTCCATGC-3'</td>
<td>5'-TTCCAGCTCTGGGATGACCTT-3'</td>
</tr>
</tbody>
</table>
incubated with secondary antibodies for 2 h at room temperature. The blots were visualized by ECL kit.

Statistical analysis

Each experiment was repeated at least three times, and all quantitative data were expressed as mean ± SD. Statistical differences between the control and experimental groups were analyzed by analysis of variance (ANOVA) and Student's t-test using IBM SPSS Statistics 22.0. Values of $P < 0.05$ were considered statistically significant.

Results

_Inhibition of osteoclast differentiation and bone-resorbing activity by CEE_

CEE (0.025, 0.05, 0.1, 0.125, 0.15 and 0.3 mg/mL) had no effect on the proliferation of BMMs (Figure 2a). CEE at various concentrations (0.075 mg/mL, 0.15 mg/mL, 0.3 mg/mL) were used in the further research. Osteoclasts were induced from mouse BMMs in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL) to verify the effect of CEE on osteoclastogenesis. After TRAP staining, the mature TRAP-positive multinucleate osteoclasts were dyed wine red (Figure 2b). Compared with blank group, the number of mature osteoclasts increased notably in RANKL-induced group. While CEE reduced the formation and numbers of osteoclasts in a dose-dependent manner ($P < 0.001$, Figure 2c).

We also investigated the effects of CEE on the bone-resorption activity of mature osteoclasts. 0.15 mg/mL CEE has no effect on osteoclast survival (Figure 2a) but substantially

![control test result graphs](image-url)
Reduced the resorption pits area ($P < 0.01$, Figure 2d-e), suggesting that CEE decreased the bone resorption activity of mature osteoclasts without a reduction in osteoclast viability. **CEE suppressed the expressions of MMP-9, Cathepsin K and TRAP of osteoclasts**

The levels of MMP-9, Cathepsin K and TRAP in osteoclasts was detected by qRT-PCR and WB (Figure 3). The gene expressions of Ctsk, Mmp-9 and Trap decreased significantly in 2-4 d, the expressions of Ctsk and Mmp-9 genes in CEE-treated groups and E$_2$ group decreased significantly on the 6th day (Figure 3a). Compared with DMSO group, the expressions of osteoclast marker proteins in 0.3 mg/mL CEE group were significantly lower (MMP-9: $P < 0.001$, Cathepsin K: $P < 0.01$, TRAP: $P < 0.001$). The results showed that CEE could significantly inhibit the expressions of MMP-9, Cathepsin K and TRAP in osteoclasts.

**CEE inhibited the expressions of RANK, TRAF6, c-Fos and NFATc1 of osteoclasts.**

Rank gene expression in 0.075 mg/mL CEE group was significantly lower than DMSO group and 0.3 mg/mL CEE group ($P < 0.05$). Compared with 0.075 mg/mL CEE group, Rank gene expressions in E$_2$ group and 0.15 mg/mL CEE group were higher, but there was no statistical significance ($P > 0.05$, Figure 4a). Erα gene expressions in 0.3 mg/mL CEE group were dose-dependent. The results showed that CEE could significantly inhibit the gene expressions of Ctsk, Mmp-9 and Trap.

As shown in Figure 3b, the protein expressions of MMP-9, Cathepsin K and TRAP in DMSO group were significantly higher than those in the blank group (MMP-9: $P < 0.001$, Cathepsin K: $P < 0.01$, TRAP: $P < 0.001$). Compared with DMSO group, the expressions of osteoclast marker proteins in 0.3 mg/mL CEE group were significantly lower (MMP-9: $P < 0.001$, Cathepsin K: $P < 0.05$, TRAP: $P < 0.001$). The results showed that CEE could significantly inhibit the expressions of MMP-9, Cathepsin K and TRAP in osteoclasts.

**Fig. 3** CEE significantly inhibited the expressions of MMP-9, Cathepsin K and TRAP in osteoclasts. (a) Effect of CEE on gene expressions of Ctsk, Mmp-9 and Trap. (b) WB bands of TRAP, Cathepsin K, MMP-9 and GAPDH. (c) Protein expressions of TRAP, Cathepsin K and MMP-9. n = 3. Compared with DMSO group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $P < 0.01$, ***$P < 0.001$, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $P < 0.05$, $P < 0.01$. **$P < 0.001$, $P < 0.01$. **$P < 0.001$. **$P < 0.001$. **$P < 0.001$. **$P < 0.001$. **$P < 0.001$. **$P < 0.001$. **$P < 0.001$.
CEE group and E2 group were significantly higher than that in DMSO group (0.3 mg/mL CEE group: \( P < 0.001 \), E2 group: \( P < 0.01 \)). E\( \alpha \) gene expression in 0.3 mg/mL CEE group was significantly higher than in 0.15 mg/mL, 0.075 mg/mL CEE group and E2 group (0.15 mg/mL, 0.075 mg/mL CEE group: \( P < 0.001 \), E2 group: \( P < 0.01 \), Figure 4b). Compared with DMSO group, Nfatc1 gene expressions in CEE-treated groups were significantly lower (\( P < 0.001 \), Figure 4c).

As shown in Figure 4d-e, the protein expressions of RANK, E\( \alpha \), c-Fos and NFATc1 in DMSO group were significantly higher than those in blank group (RANK: \( P < 0.001 \), E\( \alpha \), c-Fos and NFATc1: \( P < 0.01 \)). E\( \alpha \) protein expressions in DMSO group and CEE-treated groups were significantly higher than that in the blank group. Compared with DMSO

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**Figure 4**  CEE inhibited the expressions of key signaling molecules of osteoclasts. (a) Gene expression of Rank. (b) Gene expression of E\( \alpha \). (c) Gene expression of Nfatc1. (d) WB bands of RANK, E\( \alpha \), TRAF6, c-Fos, NFATc1 and GAPDH. (e) Effect of CEE on protein expressions of key signaling molecules. \( n = 3 \). Compared with DMSO group, *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \); Compared with 0.3 mg/mL CEE group, *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \).
group, there were no significant differences in ERα protein expressions in CEE-treated groups, suggesting that CEE inhibited osteoclast differentiation may not via promoting the expression of ERα. The protein expressions of RANK, c-Fos and NFATc1 decreased significantly in 0.3 mg/mL CEE group (RANK and NFATc1: \( P < 0.001 \), c-Fos: \( P < 0.01 \)), and the expression of TRAF6 decreased significantly in 0.3 mg/mL CEE group (\( P < 0.05 \)), but there was no statistical significance in 0.15 mg/mL CEE group. The results showed that CEE could significantly inhibit the protein expressions of RANK, TRAF6, c-Fos and NFATc1.

**CEE inhibited the phosphorylation levels of ERK, JNK and p65**

After adding drugs for 4 hours, each group was stimulated with RANKL for 0, 5, 15, 30 and 60 minutes respectively to detect the effect of CEE on total and phosphorylation protein levels of key molecules in different signal pathways.

As shown in Figure 5, compared with DMSO group, CEE significantly inhibited the phosphorylation level of JNK at 15 min (\( P < 0.001 \)), the phosphorylation levels of ERK at 5 min, 30 min and 60 min (5 min: \( P < 0.001 \), 30 min and 60 min: \( P < 0.05 \)) and the phosphorylation of p65 at 0 min, 5 min and 15 min (\( P < 0.05 \)), but there was no significant difference at other point of time (\( P > 0.05 \)). The phosphorylation levels of IκBα, p38 and Akt had no significant change compared with DMSO group at all time points. Compared with DMSO group, the total protein levels of JNK increased...
significantly at 0 min and 30 min (P < 0.05), p65 and Akt increased significantly at 0 min in CEE group (p65: P < 0.001, Akt: P < 0.05), and the total protein levels of other signal molecules had no significant change. It suggested that CEE could restrain osteoclast differentiation by inhibiting ERK, JNK and p65 phosphorylation, and not by inhibiting the total protein expressions.

CEE inhibited ovariectomy-induced bone loss

Bone mass in OVX-induced osteoporosis mouse was analyzed by micro-CT. Region of interest and longitudinal section were shown in Figure 6b. As Figure 6a, Figure 6c and Figure 6d showed, in OVX group, bone mass was significantly less than those in Blank and SHAM group, the bone trabeculae became thinner and less. After CEE treatment, there were thicker and more bone trabeculae, bone mass was increased.

BV/TV in OVX-HD group was significantly higher than that in OVX group (P < 0.05, Figure 6e). In OVX-LD and OVX-E2 groups, BV/TV also displayed positive results (P > 0.05). In Figure 6f, Tb.Th in all CEE-treated and OVX-E2 groups were significantly higher than that in OVX group (OVX-HD group: P < 0.05, OVX-LD group: P < 0.01, OVX-E2 group: P < 0.001, respectively). BV/TV and Tb.Th in OVX-HD group were higher than in OVX-LD but there were no differences. In Figure 6g and Figure 6h, BS/BV and SMI in CEE-treated groups were significantly lower than in OVX group (P < 0.001, P < 0.05, respectively). Compared to the OVX group, the CEE-treated groups exhibited no differences in BV, BMC and BS/TV but increased gradually (P > 0.05, Figure 6i-k).

CEE increased the maximal loads of tibias from OVX mice

The maximum load pressure was tested by three-point bending test. In Figure 6l, the maximal load of tibia in OVX group was significantly lower than that of SHAM group (P < 0.05). Compared with OVX group, the maximal loads of OVX-HD, OVX-LD and OVX-E2 groups were increased significantly (P < 0.05). The maximal load of tibia in OVX-E2 group was larger than those in CEE-treated groups, but there was no statistical difference (P > 0.05), and there were no statistical interactions between OVX-HD and OVX-LD groups (P > 0.05).

CEE up-regulated E2 and OPG levels, down-regulated RANKL level in serum

The levels of RANKL, E2 and OPG in serum of mice were detected and analyzed (Figure 7a-c). Compared with OVX group, the levels of E2 and OPG in SHAM group and OVX-HD group were significantly increased (E2: P < 0.05, OPG: P < 0.01, Figure 7a, c), and the levels of RANKL were significantly decreased (P < 0.001, Figure 7b). The serum levels of RANKL, E2 and OPG in OVX-LD group were higher than those in OVX-HD group, but with no significant difference (P > 0.05). And there were no significant differences between E2 and OPG-HD groups (P > 0.05).

CEE inhibited the MMP-9, Cathepsin K and TRAP expressions of OVX mice

The levels of osteoporosis related genes in mouse femur were detected by qRT-PCR, and the protein expression levels were detected by WB (Figure 8). Expressions of TRAP, Cathepsin K, MMP-9 genes and proteins in femora of OVX group were significantly higher than those of Blank and SHAM group. Compared with OVX group, the gene and protein expressions of TRAP, Cathepsin K, and MMP-9 in CEE-treated groups and OVX-E2 group were significantly reduced. There were no significant differences in gene expressions between OVX-E2, OVX-HD and OVX-LD group (P > 0.05, Figure 8a). The protein expressions of Cathepsin K and TRAP in OVX-HD group were significantly higher than those in OVX-LD and OVX-E2 group (P < 0.001), and the
Fig. 6  Stereological parameter of mouse femur bone trabecular after CEE treatment analyzed by micro-CT and histological analysis. (a) Histological analysis of tibiae with H&E staining. (b) Region of interest (ROI) and longitudinal section. (c) 3D reconstruction pictures of ROI. (d) Intersecting surface of ROI. (e) Bone volume/total volume (BV/TV). (f) Trabecular thickness (Tb.Th). (g) Bone surface/bone volume (BS/BV). (h) Structure model index (SMI). (i) Bone surface/total volume (BS/TV). (j) Bone mineral contents (BMC). (k) Bone volume (BV). (l) The maximum loads pressure of each group. n = 6, *P < 0.05, **P < 0.01, ***P < 0.001 as compared with OVX group.
protein expression of MMP-9 in OVX-HD group was higher than those in OVX-LD and OVX-E2 group (P > 0.05, Figure 8c).

**Discussion**

With the aging of the population, the increasing number of osteoporosis patients has brought a huge economic and health burden on society. Effective prevention and treatment of osteoporosis has become an important issue that Chinese society needs to solve urgently. At present, hormone replacement therapy (HRT) and bisphosphonates are the primary therapeutic strategies for bone loss diseases including osteo-

**Fig. 7** The effect of CEE on tibia mechanical properties and levels of osteoporosis related factors in serum of mice. (a) The level of OPG in serum. (b) The level of RANKL in serum. (c) The level of E2 in serum. Each sample was repeatedly tested at least 3 times, n = 6. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with OVX group.

**Fig. 8** CEE suppressed the MMP-9, Cathepsin K and TRAP expressions of femora in OVX mice. (a) Gene expressions of Trap, Ctsk and Mmp-9 in femora. (b) WB bands of TRAP, Cathepsin K, MMP-9 and GAPDH. (c) Protein expressions of TRAP, Cathepsin K and MMP-9 in femora. Each sample was repeatedly tested at least 3 times, n = 6. *P < 0.05, **P < 0.01, ***P < 0.001 as compared with OVX group.
porosis [35], but they have limited therapeutic effects and many adverse reactions. Traditional Chinese medicine has become increasingly popular due to its effectiveness in treating diseases, with fewer side-effects [36]. Recent scientific reports suggest that many natural herbal therapies have both anabolic and antacatabolic effects for the treatment of osteoporosis by promoting bone formation and reducing unbalanced bone resorption [37, 38]. Studies have found that cyasterone, as the main active component of Cyathulae Radix, treated osteoporosis by inhibiting osteoclastic differentiation and promoting osteogenic differentiation [37].

Excessive activation of osteoclast activity is responsible for many bone diseases, such as osteoporosis, rheumatoid arthritis, periarticular osteolysis, and periodontitis [39]. Natural Chinese medicine can promote bone formation activity and significantly inhibit bone resorption activity, thereby potentially alleviating the imbalance between bone formation by osteoblasts and bone resorption by osteoclasts [37]. Recently, targeting osteoclasts has gradually become the first-line therapeutic strategy in osteoporosis treatment. Given the decisive role of osteoclasts in bone resorption, molecular regulators of osteoclast formation and function are an important area of research in the treatment of osteoporosis [40]. Therefore, the present study investigates the effect and mechanism of CEE on inhibiting osteoporosis and osteoclastogenesis from the three levels of animal model, cell culture and molecular biology based on ER/RANK/NFATc1 signaling pathway.

In ER/RANK/NFATc1 signaling pathway, estrogen affects bone metabolism mainly by binding with ERα. ERα can inhibit the expression of RANK. Here, we found that CEE can significantly inhibit the protein expression of RANK and TRAF6 in osteoclasts, but has no significant effect on the protein expression of ERα. Furthermore, after RANKL combination with RANK, TRAF6 binds to cytoplasmic region of RANK and activates the downstream signaling pathways including NF-kB, MAPK and c-srC-P3K-Akt. MAPK signaling pathway includes ERK pathway, JNK pathway and p38 pathway. The results showed that CEE significantly inhibited the phosphorylation of ERK, JNK and p65. The signal transmitted to the downstream to activate regulatory molecules including c-Fos and NFATc1. Previous studies revealed that NFATc1 is the main regulator of osteoclast formation, which maintains high expression level through self-amplification. It together with activator protein (AP - 1) [41], induced osteoclasts to express bone resorption related proteins, including CTSK, MMP9, and TRAP [42]. Our data suggested that CEE treatment decreased the transcriptional activity of NFATc1 - 1 and its related gene expression including c-Fos, MMP-9, Cathepsin K, and TRAP.

In vivo, ovariectomized mice were used in this study to verify the anti-osteoporosis activity of CEE. Here, after O VX mice were treated with CEE, the bone microstructure was improved, bone loss was reduced, bone mass, strength, the thickness and quantity of bone trabecula were also enhanced. In addition, the genes and proteins expression of TRAP, Cathepsin K, MMP-9 in O VX mice were decreased by CEE. These results confirmed the anti-osteoporotic activity of CEE. Previous research found that the combination of RANKL and its receptor Rank can activate osteoclasts. On the other hand, OPG secreted by osteoblasts blocks RANK/RANKL interaction by binding to RANKL, thereby preventing osteoclast differentiation and activation [43]. In this study, we found that CEE also up-regulated the contents of E2 and OPG, down-regulated the content of RANKL in serum. The ratio of these products plays a key role in bone remodeling, which may modulate the ability of osteoblasts to stimulate the differentiation and activation of osteoclasts, as well as the degree of bone resorption. It was suggested that CEE may regulate the expression of OPG/RANKL, and then regulate the lineage of osteoblast and other stromal cells, thereby the expression of osteoclasts and treating osteoporosis. Next, it is very necessary to explore the role of CEE in the coupling ability of osteoclasts and osteoblasts.

Collectively, our study demonstrated that CEE could inhibit the differentiation and activity of osteoclasts induced by RANKL. The effect of CEE on RANKL - mediated ERK, JNK, NF - kB, and other downstream factors in osteoclast or the downstream inhibitory effect is at least in part due to its inhibitory effect of TRAF6. Our research also has limitations, further, we will focus on exploring the mechanism of interaction between CEE on osteoblasts and osteoclasts. Besides this, our in vitro findings demonstrate that CEE could be a potential therapy for osteoporosis and other osteolytic diseases (Figure 9).

Conclusions

CEE can significantly inhibit ovariectomized-induced osteoporosis of mice, RANKL-induced osteoclast differentiation and its bone resorption function. Based on ER/RANK/NFATc1 signaling pathway, CEE suppressed the expression of RANK, TRAF6, c-Fos, NFATc1, the phosphorylation of ERK, JNK in MAPK pathway and the phosphorylation of p65 in NF-kB pathway to inhibit the osteoclast differentiation and bone resorption function.

Data Availability Statement:
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations

BMC: Bone mineral content; BMMs: Bone marrow macrophages; BS: Bone surface; BSA: Bovine serum albumin; BV: Bone volume; CEE: Cyathulae radix ethanol extract; DMSO: Dimethylsulfoxide; E2: Estriol; ERK: Extracellular regulated protein kinases; ERα: Estrogen receptor α; JNK: c-Jun N-terminal kinase; M-CSF: Macrophage colony stimulating factor; MMP-9: Matrix metalloprotease 9; NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1; OPG: Osteoprotegerin; O VX: Ovariectomy; PVDF: Polyvinylidene
fluoride; RANK: Receptor activator of nuclear factor-κB; SMI: Structure model index; Tb.Th: Trabecular thickness; TRAF6: TNF receptor associated factor 6; TRAP: Tartrate resistant acid phosphatase; TV: Total volume.

Institutional Review Board Statement:

The care and experimental protocol of this study were approved by the Animal Experimentation Ethics Committee of Central South University (Ref. No.2018sydw0207) and conducted according to the ethical standards and national guidelines.

References


