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Water-soluble curcuminoids-rich extracts suppress bone resorption by inhibiting osteoclastogenesis in RANKL-induced raw 264.7 macrophages

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ABSTRACT

Background: To determine the osteoclastogenesis inhibitory effect and inside mechanisms of a curcuminoids-rich extract (CRE) and three water-soluble CREs, namely CRE-SD (CRE in solid dispersion form), CRE-Bin (CRE binary complex with hydroxypropyl-β-cyclodextrin), and CRE-Ter (CRE ternary complex with hydroxypropyl-β-cyclodextrin and polyvinylpyrrolidone K30), as well as curcumin (Cu), bisdemethoxycurcumin (Bis), and demethoxycurcumin (De).

Methods: TRAP (tartrate-resistant acid phosphatase) and acid phosphatase assays were used to confirm osteoclastogenesis. MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] and LDH (lactate dehydrogenase) assays were performed to evaluate cell viability and cell cytotoxicity, respectively. Mechanisms of action and signaling pathways were explored using Western blot analysis. Bone degradation was measured using a bone pit and fluorescein assays.

Results: A reduction in both TRAP content was demonstrated, along with lower levels of the osteoclast-specific genes cathepsin K, c-Fos, and NFATc1 (Nuclear factor of activated T cells 1), as a consequence of the effects of CRE-Ter, CRE-Bin, CRE-SD, CRE, and Cu. Exposure to CRE-Ter, CRE-Bin, and CRE-SD at 20 µg/ml and CRE and curcumin at 5 µg/ml, respectively, prevented multinuclear formation, increased acid phosphatase granule content, and reduced TRAP activity in RANKL (receptor activator of nuclear factor κ-B ligand)-treated RAW 264.7 cells. Furthermore, all curcuminoids could suppress the canonical RANKL-induced NF-κB (nuclear factor kappa B) pathway component, p-NF-κB p65 protein, while the bone pit assay confirmed that bone degradation could be reduced by curcuminoids, while the cell fluorescence content could also be lowered.

Conclusion: It was determined through the tests carried out in this research that the principal inhibitors of bone resorption and osteoclastogenesis were CRE-Ter, CRE-Bin, CRE- SD, CRE, and Cu.

KEYWORDS

Curcumin; Curcuminoid; Inclusion complex; Solid dispersion; Ternary complex

ARTICLE HISTORY

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Introduction

Osteoporosis is one of the most common diseases worldwide among the elderly, especially post-menopausal women, which decreases bone density and leads to easily fractured bones. In the over-50 age group, just over 30% of females and 20% of males who have osteoporosis will see the condition result in bone fractures [1]. The main cause of osteoporosis is the deficiency of estrogen based on the increasing number and activity of osteoclasts [2]. Moreover, deficiency of vitamin D, calcium, and hyperthyroidism are also causing osteoporosis [3]. Approval has been granted by the Food and Drug Administration (FDA) for calcitonin, bisphosphonate, estrogen and hormone replacement therapy to be used to treat osteoporosis [4]. However, some drugs can cause adverse effects; for example, bisphosphonates can cause severe musculoskeletal pain, esophageal cancer, ocular inflammation, and upper gastrointestinal injuries [5,6]. Additionally, bisphosphonates reportedly exhibit gastrointestinal interactions when used in combination with nonsteroidal anti-inflammatory drugs [7]. Therefore, natural compounds should be investigated as another option for treating osteoporosis.

Many natural compounds are good choices for prevention and treatment, as they can remedy bone resorption with little or no adverse effects. For example, the influence of *Anoectochilus formosanus* on RAW 264.7 cells was to lower RANKL (receptor activator of nuclear factor κ-B ligand) expression; therefore, it inhibited osteoclastogenesis [8]. *Acanthopanax senticosus* increased osteocalcin, which acts as a marker for bone formation; in contrast, it served to lower the C-terminal telopeptide of type-1 collagen, which acts as a marker for bone resorption [9]. Furthermore, studies have shown that aloin can strongly inhibit bone resorption. It can

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inhibit protein synthesis in the NF-κB signaling pathway, involving the proliferation and differentiation of RAW 264.7 cells into osteoclasts, thus leading to a decline in the osteoclast markers (TRAP) and cathepsin K [10]. Berberine can inhibit osteoclastogenesis by lessening RANK/RANKL/OPG pathways that cause high expression of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and osteoprotegerin (OPG) and less expression of osteocalcin, serum alkaline phosphatase (ALP), methylenedioxyamphetamine (MDA), and RANKL [11].

Turmeric (*Curcuma longa* L.) can be found across Southeast Asia, including Thailand. Turmeric has many scientifically confirmed pharmacological activities, including anti-oxidation, anti-allergy, anti-inflammation, anti-bacterial, and anti-cancer [12,13]. Curcumin (Cu), bisdemethoxycurcumin (Bis), and demethoxycurcumin (De) are the principal bioactive curcuminoids in turmeric [12], while osteoporosis has already been treated by curcumin [14]. Curcumin has exhibited anti-osteoclastogenesis by increasing the expression of glutathione peroxidase 1 (GPX-1), reducing homocysteine expression, lessening GPX-1 expression, and suppressing NF-κB [15,16]. When used alongside piperine, curcumin has the effect of suppressing osteoclastogenesis [17] and significantly increasing bone density in subjects with low bone density or osteopenia [18]. Studies of curcuminoids in both animals and humans have shown them to be non-toxic even when the dosage exceeds 12 g per day [19,20], but as a consequence of their poor gastrointestinal absorption due to their poor water-solubility [21], a very high rate of metabolism in the liver and intestines [22], and poor chemical stability when placed in an alkaline medium [23], approval has not yet been granted for their therapeutic use. Furthermore, they are unable to access the blood in adequate concentrations, which would suffice to influence disease markers or clinical end-points, even if the dosage reaches 12 g per day [20]. It is possible to reverse certain disease conditions in the case of human colon cancer when curcumin is employed at a concentration of 12.9 μg/ml [24], while a concentration of 35.1 μg/ml will generate radical scavenging activity [25], and a concentration of 9.2 μg/ml is effective for human pancreatic alpha-amylase [26]. However, the curcumin level is very low to the point of being undetectable (0.006 μg/ml at 1 h in serum level) when the human intake dosage is 2 g [27]. When the dosage was increased up to 8 g daily for 3 months, the respective level of serum curcumin was only 0.65 μg/ml [19]. Thus, curcuminoids have been concentrated to increase absorption [28].

This research examined the influence of purified curcuminoids, namely Cu, De, and Bis, CRE (curcuminoids-rich extract) containing 89.5% w/w curcuminoids, and three highly water-soluble CREs, i.e., CRE-SD (CRE in solid dispersion form with polyvinylpyrrolidone K30 or PVP-K30), CRE-Bin (CRE binary complex with hydroxypropyl-β-cyclodextrin or HPβ CD), and CRE-Ter (CRE ternary complex with HPβCD and PVP-K30). We evaluated the inhibitory capacity of these curcuminoids against osteoclastogenesis through the use of a receptor in RANKL-induced macrophage RAW 264.7 cells. It is anticipated that the research findings may support the development of these compounds for a therapeutic role in treating osteoporosis.

Materials and Methods

Chemicals

R&D Systems (Minneapolis, USA) supplied RANKL, while Gibco (Bangkok, Thailand) supplied the RPMI 1640 with 2 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS), and Antibiotic-Antimycotic (100X). Sigma (St. Louis, MO, USA) provided the Acid Phosphatase, Leukocyte (TRAP) Kit, while Cell Signaling Technology (Beverly, MA, USA) supplied the alendronate sodium trihydrate and the anti-phospho-NF-κB p65 and anti-β-actin. RNeasy Kit was obtained from Qiagen (Valencia, CA, USA), while all other unspecified reagents were purchased from Sigma or Wako Pure Chemical Industries (Bangkok, Thailand).

Preparation of curcuminoids

Cu, Bis, and De were purified from *C. longa* extract using the previously reported method [29]. CRE containing 89.5% w/w curcuminoids (72.8% Cu, 12.5% De, and Bis 4.2% w/w) was prepared from the rhizomes of *C. longa* via the approach proposed by Lateh et al. [30] involving green extraction and fractionation. The obtained CRE was subsequently used to prepare CRE-SD (CRE in solid dispersion form with PVP-K30, containing 7% w/w curcuminoids), CRE-Bin (CRE binary complex with hydroxypropyl-β-cyclodextrin, containing 17% w/w curcuminoids), and CRE-Ter (CRE ternary complex with HPβCD and PVP-K30, containing 14% w/w curcuminoids) using the previously described methods [31,32]. Water-solubility values of CRE, CRE-SD, Cre-Bin, and CRE-Ter were 0.0, 28.0, 55.6, and 70.3 μg/ml, respectively.

Cell cultures

CLS Cell Lines Service GmbH (Eppelheim, Germany) supplied macrophage RAW 264.7 cells. These cells underwent culturing in RPMI 1640 cell culture medium, which was supplemented with 2 mM glutamine, 10% heat-inactivated FBS, and Antibiotic-Antimycotic (100X) under humid 5% CO_2 and 37°C conditions. Dimethyl sulphoxide (DMSO) was used to dissolve the curcuminoid extracts before storage at 1 mg/mL for one week at -20°C. The RAW 264.7 cells were then transferred into a 6-well plate (3×10^3 cells/ well) and incubated with 20 ng/ml RANKL solution for 5 days for differentiation of the cells to be osteoclasts.

TRAP assay to confirm osteoclastogenesis

The macrophage RAW 264.7 cells were treated with or without 20 ng/ml RANKL solution for 1, 2, 3, 4, and 5 days, respectively, under humid 5% CO_2 and 37°C conditions. Upon completion of each designated period, osteoclastogenesis was determined using the Takara Bio TRAP assay kit (Tokyo, Japan). The results were expressed as the percentages of TRAP activity *vs.* days.

Assay for cell viability and cytotoxicity

Initially, a 96-well plate was used for seeding the RAW 264.7 cells (5×10^3 cells/well) either in with or without 20 ng/ml RANKL solution and with or without Cu, Bis, De, CRE, CRE-Bin, CRE-SD, and CRE-Ter at various concentrations of 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 10, 20, and 50 μg/ml. This was followed by culturing for 5 days. The proliferation of osteoclasts could then be assessed after the addition of the MTT reagent through the use of spectrophotometry at 570

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nm. The LDH reagent was then introduced to conduct the cytotoxicity assay via spectrophotometry at 490 nm [10].

Acid phosphatase, leukocyte (TRAP) activity

A 12-well plate was seeded with RAW 264.7 cells (1×10^4 cells/well) for 5 days either with or without 20 ng/ml RANKL solution, and with or without Cu (5 µg/ml), Bis (2.5 µg/ml), De (2.5 µg/ml), CRE (5 µg/ml), CRE-SD (20 µg/ml), CRE-Bin (20 µg/ml), and CRE-Ter (20 µg/ml). The TRAP assay was conducted using an acid phosphatase kit. A purplish to dark red acid phosphatase staining granule was observed in the cytoplasm of most leukocytes when monitored using a light microscope [10].

RNA isolation and real-time polymerase chain reaction

Isolation of total RNA was accomplished using ISOGEN (Nippon Gene, Toyama, Japan), while the synthesis of cDNA (complementary DNA) was performed using the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). The first strand cDNA was synthesized using RNA (1 µg) through the miScript® II RT Kit (Qiagen), where upon the synthesized product was employed for the miScript primer assays along with the Quanti Fast SYBR Green PCR Kit (Qiagen) to conduct real-time PCR for the assessment of NFATc1 (Nuclear factor of activated T cells 1), c-Fos, and cathepsin K, for which the ABI Prism 7500HT sequence detection (Applied Biosystems, CA, USA) was required. The endogenous control for these processes was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The Image Quant TL (GE HealthCare Life Sciences, Japan) alongside an Image Quant LAS 4000 (Fujifilm, Okayama, Japan) was used to determine the target genes' relative expression against GAPDH.

The following primers [33] were used in the process:

c-Fos:
Forward; CCAGTCAAGAGCATCAGCAA
Reverse; AAGTAGTGCAGCCCGGAGTA
NFATc1:
Forward; CCGTTGCTTCCAGAAAATAACA
Reverse; TGTGGGATGTGAACTCGGAA
Cathepsin K:
Forward; ATGTGGGGGCTCAAGGTTCTG
Reverse; CATATGGGAAAGCATCTTCAGAGTC
GAPDH:
Forward; AAATGGTGAAGGTCGGTGTG
Reverse; GAATTTGCCGTGAGTGGAGT

Western blotting

Over 5 days, macrophage RAW 264.7 cells were treated with or without 20 ng/ml RANKL solution and with different curcuminoid extracts, such as Cu, CRE, CRE- SD, CRE-Bin, and CRE-Ter. NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Washington DC, USA) were used to extract cytoplasmic and nuclear proteins. An SDS-PAGE gel was used to separate the proteins, which were then introduced to the polyvinyl difluoride (PVDF) membrane. The PVDF membrane was subsequently rinsed thoroughly and incubated with phospho-NF-κB p65 and β-Actin antibodies (Cell Signaling, MA, USA). The membrane was then washed again with a buffer and probed using a secondary antibody conjugated for one hour with horse radish peroxidase. The results were then obtained through a digital imaging system. Finally, a Bio-Rad Chemi Doc MP Imaging System (Bangkok, Thailand) was employed to detect the protein bands on the membrane [33].

Bone pit assay

To measure the extent of bone degradation, a bone resorption assay plate 24 (PG Research, Tokyo, Japan) was employed [34]. Initially, the macrophage RAW 264.7 cells were stored in RPMI 1640 under 5% CO_2 humid conditions at 37°C prior to the assay. The cells (1×10^3 cells/ml) were inoculated upon a bone resorption assay plate 24 before incubation in RPMI 1640 growth medium (3 ml) with 2 mM glutamine, 10% heat-inactivated FBS, 100 µg/ml streptomycin sulfate, and 100 U/ml penicillin G without phenol red under 5% CO_2 humid conditions at 37°C in the presence of 20 ng/ml RANKL solution and with Cu (5 µg/ml), CRE (5 µg/ml), CRE-SD (20 µg/ml), CRE-Bin (20 µg/ml), or CRE-Ter (20 µg/ml). The positive control for the prevention of osteoclastogenesis was alendronate sodium trihydrate (2 nM). Having completed the 21-day incubation period, sterile PBS was used to wash the plates, which were then treated for 5 minutes with 5% sodium hypochlorite. The plates were further washed in tap water and dried before photographs were taken of five different regions from inside each of the wells via microscopy (Olympus, Tokyo, Japan).

Fluorescein assay

The macrophage RAW 264.7 cells (1×10^3 cells/ml) were initially cultured in 1 ml of growth medium containing phenol red in a bone resorption assay plate 24 with 20 ng/ml RANKL solution, and with Cu (5 µg/ml), CRE (5 µg/ml), CRE-SD (20 µg/ml), CRE-Bin (20 µg/ml), or CRE-Ter (20 µg/ml). Having completed the 21-day period, the culture supernatant (100 µl) was placed in a 96-well plate, and 0.1 N NaOH (50 µl) was introduced. Measurements of the fluorescence intensity at 485 nm (excitation) and 535 nm (emission) were taken using a fluorescence plate reader (DTX 800/880 Series Multimode Detector, CA, USA). The results were shown in terms of relative intensity. Alendronate sodium trihydrate (2 nM) was used as a positive control [10].

Data analysis

The experimental components of the study were performed in triplicate. Data are presented as mean ± standard deviation. A one-way ANOVA with a post hoc Dunnett's test was employed to analyze the statistical differences among values using SPSS and was considered as a significant difference when $p<0.05$.

Results and Discussion

RANKL-induced osteoclastogenesis

Differentiation of RAW 264.7 cells to osteoclasts is characterized by the acquisition of mature phenotypic osteoclast markers, i.e., expression of TRAP, which reduces the activity of acid phosphatase in the differentiated cells, expression of cathepsin K, and morphological change into large multinucleated cells [10]. In this study, a time-course evaluation of RANKL action over 5 days was carried out to confirm the differentiation. The results revealed that the differentiation of RAW 264.7 cells to osteoclasts was observed by a significant increase ($p<0.05$) of TRAP in 3 days (Figure 1).

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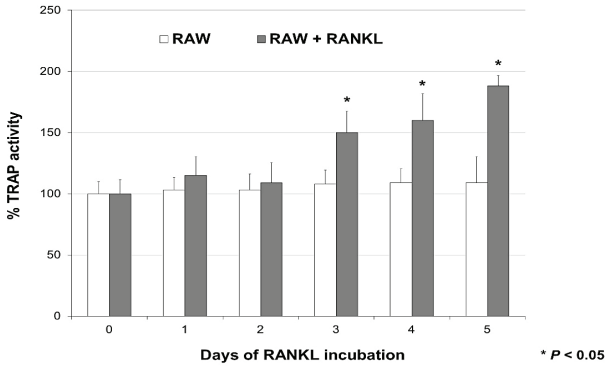

Figure 1. Osteogenesis test. Semi-confluent RAW 264.7 cells were treated with or without RANKL (20 ng/ml) for different time durations. Osteoclast marker protein TRAP was measured at 0, 1, 2, 3,4 and 5-days as per the standard method. Values are depicted as a percentage of TRAP content in the cells.

Effect of curcuminoids on cell viability and cytotoxicity

Based on cell viability and cytotoxicity assays, exposure of the RAW 264.7 cells to curcuminoid extracts with various concentrations revealed that the maximum concentrations of curcuminoid extracts that did not exhibit any cell cytotoxicity were 2.5 µg/ml for De and Bis, 5 µg/ml for Cu and CRE, and 20 µg/ml for CRE-SD, CRE-Bin, and CRE-Ter (Figures 2A and 2B). Furthermore, polyvinylpyrrolidone K30, hydroxypropyl-β-cyclodextrin for increasing the water solubility of curcuminoid extracts, and 0.25% DMSO for dissolving all curcuminoids were not toxic to the cells.

Effect of curcuminoids on osteoclastogenesis

RAW 264.7 cells without RANKL showed the presence of acid phosphatase-positive cells that produced cytosol purplish to

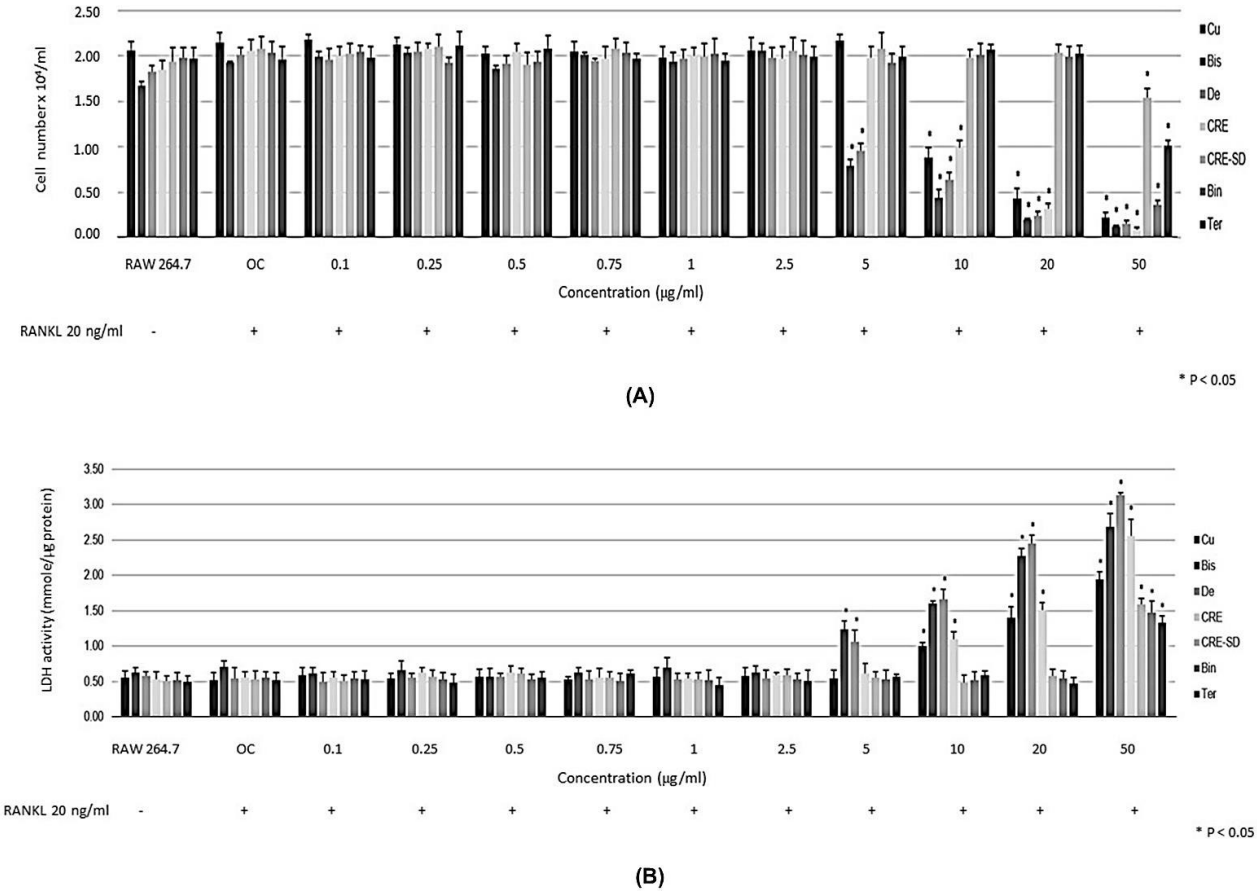

Figure 2. Cell viability based on the MTT assay (A); cytotoxicity based on the LDH activity test (B). The results are expressed as the mean ± standard deviation from six independently conducted trials. The statistical significance was set to $p<0.05$ when compared to the control.

dark red granules (Figure 3A1). The cells treated with RANKL revealed multi-nucleated and acid phosphatase-negative cells because of the presence of the TRAP content in the osteoclasts (Figure 3A2), while the cells treated with Cu (5 µg/ml), CRE (5 µg/ml), CRE-SD (20 µg/ml), CRE-Bin (20 µg/ml), and CRE-Ter (20 µg/ml) prevented cell differentiation to multi-nucleated cells and revealed positive results for acid phosphatase staining (Figures 3A3 and 3A6-3A9). In contrast, the cells treated with De (2.5 µg/ml) and Bis (2.5 µg/ml) have no effect on preventing the differentiation of the cells into osteoclasts (Figures 3A4 and 3A5). To confirm this phenomenon, Cu (5 µg/ml), CRE (5 µg/ml), CRE-SD (20 µg/ml), CRE-Bin (20 µg/ml) and CRE-Ter (20 µg/ml) inhibited cell differentiation to osteoclasts, leading to increased acid phosphatase activity (Figure 3B). Furthermore, treatment of the cells with Cu (5 µg/ml), CRE (5 µg/ml), CRE-SD (20 µg/ml), CRE-Bin (20 µg/ml), and CRE-Ter (20 µg/ml) inhibited cell differentiation, leading to decreased TRAP activity content (Figure 3C).

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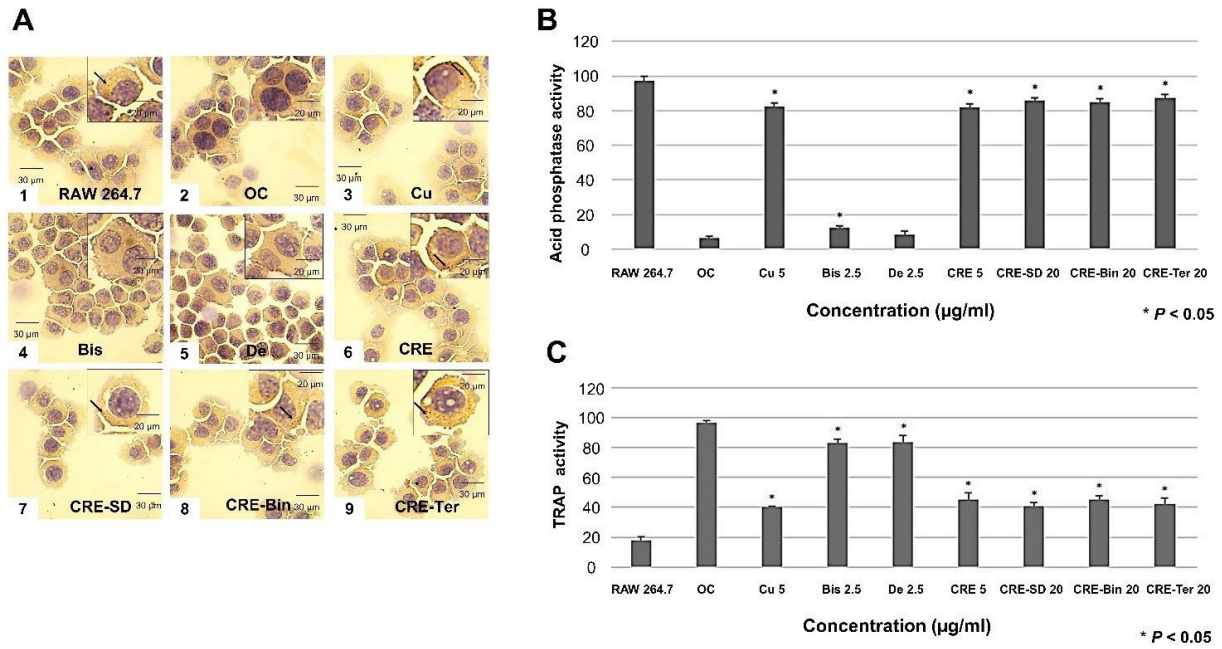

Figure 3. Differentiation of RAW 264.7 cells to osteoclasts; morphological study (A); acid phosphatase activity (B); TRAP activity (C). * indicates statistical significance (p<0.05) versus osteoclasts (OC).

Inhibition of curcuminoids on the NF-κB signaling pathway and transcription factors

Western blotting was used to find out the mechanism of inhibition of RANKL-stimulated osteoclastogenesis in RAW 264.7 cells. The results revealed that Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter dose-dependently and significantly (p<0.05) inhibited the expression of a crucial member of the NF-κB, phosphor-p65 NF-κB protein (Figure 4).

A decrease in expression of transcription factors and osteoclastic-related genes, i.e., c-Fos, NFATc1, and cathepsin K, by these curcuminoids was further determined by real-time PCR. It was found that Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter dose-dependently inhibited c-Fos, NFATc1, and cathepsin K gene expression (Figure 5). These results indicated that these curcuminoid extracts attenuated RANKL-mediated osteoclast formation by inhibiting the activation of the canonical NF-κB signaling pathway.

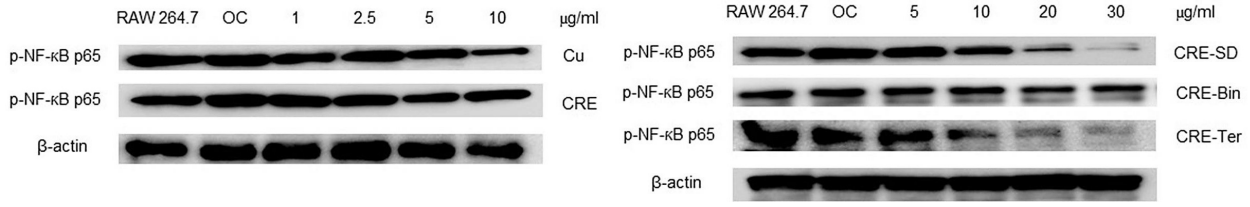

Figure 4. Effects of curcuminoid extracts, Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter on NF-Kb family pathways. RAW 264.7 cells were seeded in a 6-well plate and treated for 5 days with different concentrations of Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter and with and without RANKL (20 ng/ml). A western blot analysis of individual NF-κB members phospho-NF-κB- p65 was performed. β-Actin was used as an internal control. * indicates statistical significance (p<0.05) versus the control group.

Curcuminoids reduce the activity of osteoclasts on bone plates

The effects of the curcuminoid extracts on the pit-formation of osteoclast cells were determined, and the results are illustrated in Figure 6. The artificial bone material sheet with RAW 264.7 cells in the culture medium alone did not exhibit any pits. The cells cultured with RANKL for 21 days in the absence of Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter exhibited maximum pits (Figure 6). In RANKL-treated conditions, many resorption pits (blackish purple areas) were observed, indicating bone loss by osteoclasts. However, the pit formation of osteoclast cells was inhibited by treatments with the curcuminoid extracts (Figure 6A), indicating the efficacy of these curcuminoid extracts in the prevention of bone resorption. Finally, the prevention of bone pits by osteoclasts was determined. Results revealed the absence of bone pits in the positive control (Figure 6A), while the rate of fluorescence intensity was significantly decreased after being treated with curcuminoid extracts (Figure 6B), collectively confirming that Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter prevent bone loss on the bone plate, *in vitro*.

The balance between osteoblastogenesis and osteoclastogenesis plays an important role in bone mass homeostasis. Bone resorption is performed by the osteoclasts, which are multinucleated cells differentiating from the monocyte or macrophage cells. Homeostasis of osteoblasts and osteoclasts is principally regulated by various signaling pathways, including the RANKL/RANK signaling pathways

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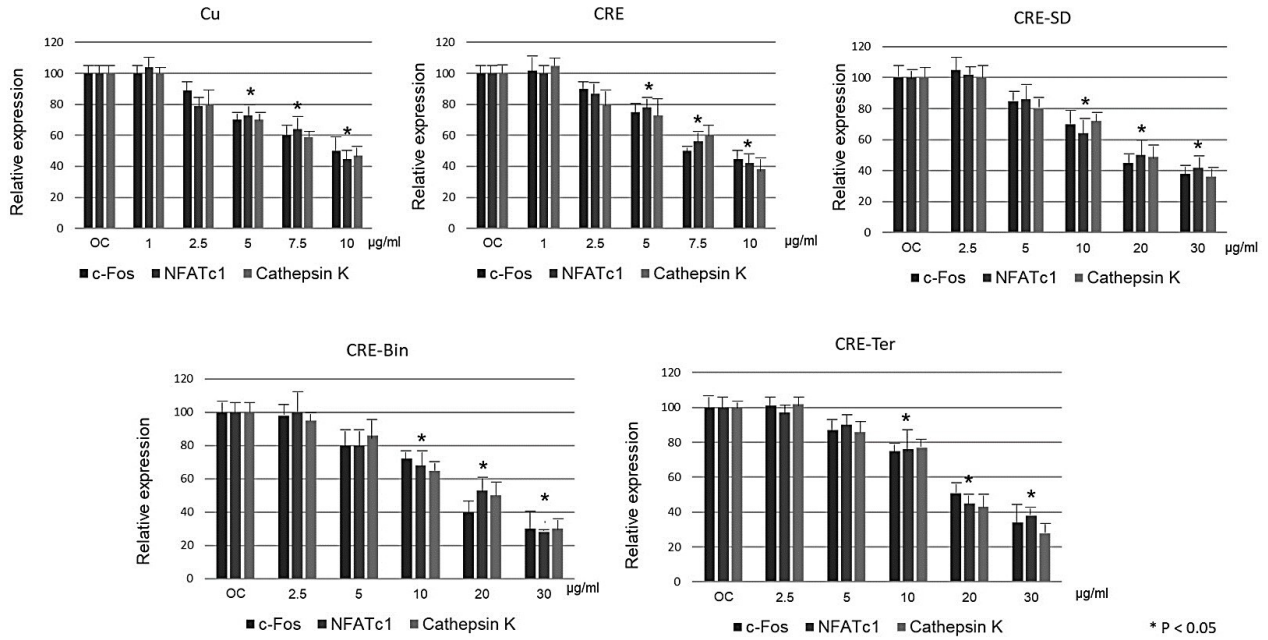

Figure 5. Effect of curcuminoid extracts on cathepsin K mRNA activity. RAW 264.7 cells, both with and without RANKL, were cultured in a 12-well plate with Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter for 5 days. Total RNA was isolated, and cDNA was synthesized using a reagent kit. Analysis of c-Fos, NFATc1, and cathepsin K by real-time PCR using the ABI Prism 7500 HT sequence detection system showed that the ratio between cathepsin K and GAPDH decreased dose-dependently. * indicates statistical significance (p<0.05) versus OC (osteoclasts).

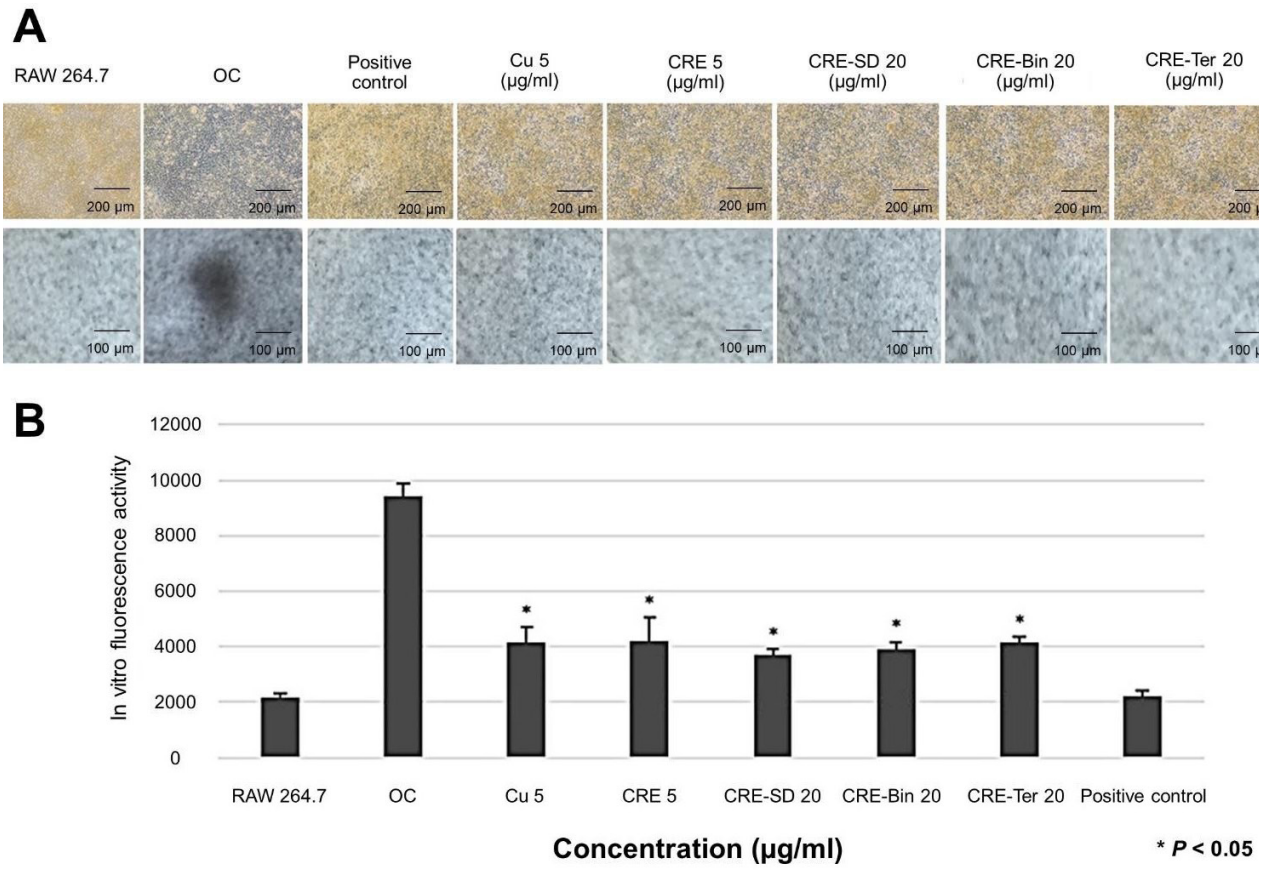

Figure 6. Bone pit assay on an artificial bone plate (A) and fluorescein activity assay of RAW 264.7 cells treated with various curcuminoids (B). * indicates statistical significance (p<0.05) versus the control group.

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that mainly rely on osteoclast differentiation. A recent understanding of osteoclast resorption mechanisms has provided new therapeutic targets for treatments of bone resorption via inhibiting the formation of osteoclasts or osteoclastogenesis. Although some anti-bone resorptive agents, including bisphosphonates, calcitonin, selective estrogen receptor modulators, and estrogen, are currently available, some of them are reportedly associated with severe adverse effects, including breast cancer, thromboembolism, atrial fibrillation, hypercalcemia, endometritis, and osteonecrosis of the jaw [35]. Moreover, some drugs cause adverse effects, such as bisphosphonate, which can cause severe musculoskeletal pain, esophageal cancer, ocular inflammation, and upper gastrointestinal injuries [5,6]. In addition, a combination of bisphosphonate with non-steroidal anti-inflammatory drugs, or NSAIDs, may cause gastrointestinal ulceration [7]. Therefore, natural compounds should be investigated as an alternative route for treating osteoporosis. It has been reported that RANKL-induced osteoclast formation is correlated with secreted TRAP activity [10]. Our results also showed that Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter decreased RANKL-induced osteoclast formation and TRAP activity, multinucleated formation, and the transcriptional osteoclast-specific gene expressions, i.e., c-Fos, NFATc1, and cathepsin K in RANKL-induced RAW 264.7 cells (Figure 5). These results imply that Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter potentially inhibit osteoclast differentiation by attenuating RANKL signaling. However, the inhibition potential is also dependent on the bioavailability of these curcuminoid extracts, which is highly related to their water solubility properties. This might be observed by the lower concentrations of total curcuminoids in CRE-SD, CRE-Bin, and CRE-Ter used for the inhibitory effect compared to those of Cu and CRE.

By inhibiting the formation of osteoclasts, these curcuminoid extracts could provide useful molecular and cellular ways to treat osteolysis. In the canonical NF-κB pathway, ligation of RANK activates the inhibition of the IκB kinase complex, leading to its ubiquitination and proteasome degradation [36], and leads to the release of NF-κB dimers containing p65 (RelA) and c-Rel into the cytosol, allowing them to translocate into the nucleus, where they enhance transcription of target genes such as TRAP, c-Fos, NFATc1, and cathepsin K [37-40]. The present study showed that phospho-NF-κB p65, the active form of NF-κB p65 (RelA) protein, which translocated into the nucleus, was decreased in a dose-dependent manner during treatment of the cells with Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter. It could be hypothesized that these curcuminoid extracts act on the RANK circuit through the NF-κB pathway. In addition, inhibition of the NF-κB pathway that is involved in the regulation of cell growth could explain the negative effects on osteoclasts, which is a key mechanism associated with anti-osteoclastogenic effects [41,42]. The present study confirms that these curcuminoid extracts inhibited phospho-NF-κB p65 protein, the crucial protein in the NF-κB pathway that was markedly induced by RANKL. Based on *in vitro* bone resorption activity, the present study also exhibited that active osteoclasts possessed characteristic membranes, including the basolateral secretory membrane, ruffled border, and attachment zone. The ruffled border has secreted protons and enzymes, including cathepsin K and TRAP, enabling the solubilization and digestion of the bone matrix after attachment to the bone. Based on the results from an *in vitro* bone pit resorption assay and a decrease in fluorescein activity, osteoclastic bone resorption was inhibited by these curcuminoid extracts (Figures 6A and 6B).

Additionally, our previous study demonstrated that CRE-Ter inhibited miR-21 gene expression in RANKL-induced RAW 264.7 macrophage via the NFκ B-Akt-miR-21 pathway [43]. CRE-Ter and CRE-SD were also recently formulated by encapsulated into the liposomes and studied for their anti-osteoclastogenesis [33,44]. These studies provide the possibility of anti-osteoclastogenic effects of both liposomal preparations through RANKL-induced RAW 264.7 cells and the development of treatments for osteolysis.

Conclusions

Our findings clearly reveal that the curcuminoid extracts, namely Cu (5 μg/ml), CRE (5 μg/ml), CRE-SD (20 μg/ml), CRE-Bin (20 μg/ml), and CRE-Ter (20 μg/ml), possess anti-osteoclastogenic potential through reducing the *in vitro* canonical RANKL-induced NF-κB signaling pathway and inhibiting transcription of c-Fos, NFATc1, and cathepsin K involved in osteoclastogenesis. This study provides further insight on Cu, CRE, CRE-SD, CRE-Bin, and CRE-Ter as inhibitors of osteoclastogenesis and *in vitro* bone resorption as new design drugs for osteolysis. However, further study on the anti-osteoporosis effects of these curcuminoid extracts is required to explore their *in vivo* therapeutic effects.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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