Bioorganic & Medicinal Chemistry 16 (2008) 9176–9183

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Aldehydic components of Cinnamon bark extract suppresses RANKL-induced osteoclastogenesis through NFATc1 downregulation

Kentaro Tsuji-Naito *

DHC Corporation Laboratories, Division 2, 2-42 Hamada, Mihama-ku, Chiba 261-0025, Japan

ARTICLE INFO

Article history:
Received 24 July 2008
Revised 5 September 2008
Accepted 6 September 2008
Available online 14 September 2008

Keywords:
Cinnamomum zeylanicum
Osteoclastogenesis
NFATc1
RANKL

ABSTRACT

Several major bone diseases are directly attributable to bone loss, including osteoporosis, bone metastasis, and rheumatoid arthritis. The nuclear factor of activated T cell 1 (NFATc1), a transcription factor, has recently been shown to play an essential role in osteoclastogenesis. In this study, we found that of several herbs, *Cinnamomum zeylanicum (C. zeylanicum)* exhibited the strong inhibitory effects on osteoclastogenesis and that its mechanism of action involves the suppression of NFATc1-mediated signal transduction. *C. zeylanicum* dose-dependently inhibited osteoclast-like cell formation at concentrations of 12.5-50 μg/ml without affecting cell viability. Resorption pit assays have shown that *C. zeylanicum* also inhibits the bone-resorbing activity of mature osteoclasts. Treatment with *C. zeylanicum* inhibited the receptor activator of nuclear factor-κB ligand (RANKL)-induced NFATc1 and c-*fos* expression. Additionally, *C. zeylanicum* moderately inhibited phosphorylation of IκB-α, suggesting that the c-*fos*/NFATc1 pathway, rather than the nuclear factor-κB (NF-κB) pathway, is the primary target of *C. zeylanicum* during RANKL-induced osteoclastogenesis. Using an HPLC-DAD system, we identified three major peaks for four characteristic components in the *C. zeylanicum* extract and identified an unknown peak as 2-methoxycinnamaldehyde via HPLC and a 2D-COSY ^{1}H NMR study. We identified cinnamaldehyde and 2-methoxycinnamaldehyde as active components reducing osteoclast-like cell formation and inhibiting NFATc1 expression. Notably, in a resorption pit assay, 2-methoxycinnamaldehyde exhibited remarkable inhibition rates of 95% at 2 μM on bone resorption. In summary, this study points to the conclusion that *C. zeylanicum* inhibits RANKL-induced osteoclastogenesis. This finding raises prospects for the development of a novel approach in the treatment of osteopenic disease.

1. Introduction

The balance between bone resorption (by osteoclasts) and bone formation (by osteoblasts) maintains bone homeostasis in a process called bone remodeling.[1] Incremental changes in the rate of bone resorption can lead to bone disruption and cause major bone diseases, including osteoporosis, bone metastasis, and rheumatoid arthritis. Understanding the mechanism that regulates osteoclastogenesis would pose significant clinical implications and raise prospects for finding ways to control this mechanism.[2]

Large tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs) that are hematopoietic in origin, osteoclasts are capable of resorbing bone.[3,4] Osteoclastogenesis progresses through multiple stages, including differentiation, fusion, and activation (maturation) regulated by various factors, including cytokines, hormones, and other cells in the bone microenvironment. A key factor is RANKL, a member of the tumor necrosis factor family.[5] RANKL mediates osteoclastogenesis by binding to its receptor RANK on osteoclast precursor cells. Analyses of RANK-deficient mice show that an intrinsic defect in the osteoclasts causes serious osteopetrosis,[6] implicating RANKL as an essential factor in modulating osteoclast differentiation and activation.

The association of RANKL with RANK recruits tumor necrosis factor receptor-associated factors such as NF-κB-inducing kinase, activating NF-κB signaling pathways.[7–10] Other essential regulatory factors include c-*fos*; c-*fos*-deficient mice also exhibit deficient osteoclast formation and have an osteopetrotic phenotype.[11] Recent reports indicate that ectopic expression of NFATc1 causes precursors to undergo efficient differentiation in the absence of RANKL and that NFATc1-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL.[12] The induction of NFATc1 is regarded as a hallmark event in the cell fate determination of osteoclasts. In a genome-wide search for the RANKL-inducible genes specifically required for terminal differentiation of osteoclasts, NFATc1 was shown to be strongly induced by RANKL. NFATc1 expression depends on the NF-κB and c-*fos* pathways activated by RANKL, suggesting NFATc1's integral role in RANKL signaling.[12]

Several recent reports whose goal is to identify patterns for preventing osteoporosis through daily diet examined the effects of food components and their bioactive components on bone metabolism.[13–17] We also focused on identifying inhibitors of osteo-

* Tel.: +81 43 275 4811; fax: +81 43 275 4831.
E-mail address: knaito@dhc.co.jp.

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.09.036

clastogenesis among medicinal food stuffs, screening an extract library prepared from 33 plants for effective dual inhibitors of both TRAP$^+$ MNC formation and NFATc1 expression in RANKL-induced osteoclastogenesis systems. In preliminary experiments, we performed a screening test using a TRAP assay with a finding that seven herbal extracts (*Chaenomeles sinensis, C. zeylanicum, Artemisia princeps, Citrus sinensis var. daidai, Crocus sativus, Citrus limon, and Mallotus japonicus*) strongly inhibit TRAP$^+$ MNC formation. We subsequently screened these herbal extracts for strong inhibitors of NFATc1 expression. Of these extracts, the molecular target-based screening tests identified *C. zeylanicum* extract as the most potent inhibitor.

Cinnamon bark or cortex is a popular natural spice and herb commonly used in traditional Chinese medicine to treat gastritis, blood circulation disturbances, and inflammatory disease.[18–20] In recent years, their pharmacological effects in the treatment of type 2 diabetes have attracted wide attention.[21] Choi et al. has also reported that *Cinnamomum cassia* (*C. cassia*) stimulates bone formation by osteoblasts in vitro,[22] but little work has been done to evaluate the effects on osteoclast differentiation and bone resorption. In our study, we demonstrate for the first time that cinnamon bark strongly inhibits osteoclastic activity and that its mechanism of action is attributable to the suppression of NFATc1-mediated signal transduction. We also identified cinnamaldehyde (CA) and 2-methoxycinnamaldehyde (2-MCA) as its active components. Our results raise the possibility that *C. zeylanicum* may be used to facilitate bone modulation in osteopenic disease.

2. Results

2.1. Cinnamon bark extract suppresses RANKL-induced osteoclastogenesis by restriction of NFATc1 expression in RAW264.7 cells

NFATc1, an essential transcriptional factor for osteoclast differentiation, is upregulated after RANKL stimulation.[12] In preliminary experiments, to explore effective inhibitors of RANKL-induced osteoclastogenesis, we investigated the effects of seven herbal extracts on both TRAP$^+$ MNC formation and NFATc1 expression. This molecular target-based screening identified *C. zeylanicum* extract as the most potent inhibitor among these extracts (data not shown). To confirm the effects of *C. zeylanicum* extract on osteoclast differentiation, we evaluated the number of TRAP$^+$ MNCs formed after RANKL stimulation in the presence or absence of the extract. As shown in Figure 1A and B, *C. zeylanicum* extract inhibited the formation of TRAP$^+$ MNCs in a dose-dependent manner at 12.5–50 μg/ml but had no effects on the growth or survival of RAWs (Fig. 1C). In addition, the morphology of *C. zeylanicum* extract-treated RAW cells was nearly identical to that of the control cells.

Next, to determine the effects of *C. zeylanicum* extract on bone resorption by mature osteoclasts, RAWs were cultured on calcium phosphate apatite-coated plates with either vehicle or with *C. zeylanicum* extract for 8 days, and the level of resorption activity was monitored using the BD BioCoat™ Osteologic™ Bone Cell Culture System. Treatment resulted in a dose-dependent depression of resorption activity. Complete inhibition was observed at concentrations of 50 μg/ml of *C. zeylanicum* extract (Figs. 1D and E). These data indicate that *C. zeylanicum* extract has the ability to reduce osteoclastic bone resorption associated with osteoclast differentiation.

As shown in Figure 2A, RANKL increased NFATc1 expression, with maximum induction at 48 h, indicating that *C. zeylanicum* extract at concentrations of 50 μg/ml completely blocks NFATc1 expression at all time points. We also observed that adding *C. zeylanicum* extract reduced NFATc1 expression in a dose-dependent manner and found that *C. zeylanicum* extract exerts potent inhibitory effects on both TRAP activation and NFATc1 induction, key events in osteoclast differentiation. These results suggest that the inhibition of RANKL-induced NFATc1 induction is the critical target of *C. zeylanicum* extract.

2.2. Effects of cinnamon bark extract on expression of c-*fos*, and on NF-κB signaling pathway in RANKL-stimulated RAW264.7 cells

To investigate the molecular mechanisms of *C. zeylanicum* extract in osteoclast precursors, we determined the level of expression of c-*fos*, essential factors in *NFATc1* gene expression. In particular, as is well-known, osteoclastogenesis requires c-*fos*, a transcription factor for AP-1.[11] As shown in Figure 3A, *C. zeylanicum* extract at 24 h after RANKL stimulation completely restrained expression of both c-*fos* and NFATc1.

We further examined the effects of *C. zeylanicum* extract on the NF-κB signaling pathway. NF-κB activation is known to progress through the activation of IκB kinase and the subsequent phosphorylation of IκB for ubiquitin-dependent degradation. The results in Figure 3B show that untreated RAW264.7 cells significantly increased phosphorylation of IκB-α and degradation of IκB-α via stimulation by RANKL. *C. zeylanicum* extract treatment barely suppressed phosphorylated IκB-α. Pretreatment of RAW264.7 cells with *C. zeylanicum* extract for 1 hour tended to reduce IκB-α phosphorylation, IκB-α degradation at higher concentrations compared to un-pretreated RANKL-stimulated controls (Fig. 3C), although phosphorylated IκB-α at 50 μg/ml of *C. zeylanicum* extract was still observed. These results suggest that the c-*fos*/NFATc1 pathway, not the NF-κB pathway, is the primary target of *C. zeylanicum* extract during RANKL-induced osteoclastogenesis.

2.3. Chemical profiling of *C. zeylanicum* extract and specification of its active components as effective inhibitors of RANKL-induced osteoclastogenesis

In several previous studies, the chemical components of *Cinnamomum* species have been clearly defined by HPLC analysis.[23,24] Figure 4A shows an HPLC chromatogram for *C. zeylanicum* extract. Using an HPLC-DAD system, we identified three characteristic peaks as cinnamyl alcohol (peak 1), cinnamic acid (peak 2), and CA (peak 3) by comparing corresponding retention times and UV spectra for the standards and by consulting the literature.[23,24] One undetermined peak (peak 4) was also detected in *C. cassia* (data not shown) and was previously treated as an unknown component.[24] This compound was not inconsistent with the peak of eugenol. Therefore, the fraction of peak 4 was collected and processed without further purification by the RP-HPLC isolation method (Fig. 4B). Structural identification of this compound isolated from *C. zeylanicum* extract was characterized as 2-MCA by ^{1}H NMR and 2D-COSY (Fig. 4C) and confirmed by comparison against the ^{1}H NMR spectra of the standard. 2-MCA has already been isolated and purified from cinnamon bark extract with preparative thin-layer chromatography.[25–27] However, this data may prove useful in a systematic study involving authentication and assessment of the related *Cinnamomum* species, since we determined the HPLC profile for 2-MCA, in addition to well-defined compounds.

To determine the active component(s) of *C. zeylanicum* extract, we investigated the effects of four components on both NFATc1 expression and TRAP activity. As shown in Figure 5A, CA and 2-MCA strongly inhibited NFATc1 expression in 48 h cultures of RAW cells, implying that these compounds are the active components in *C. zeylanicum* extract and that the inhibitory effects of 2-

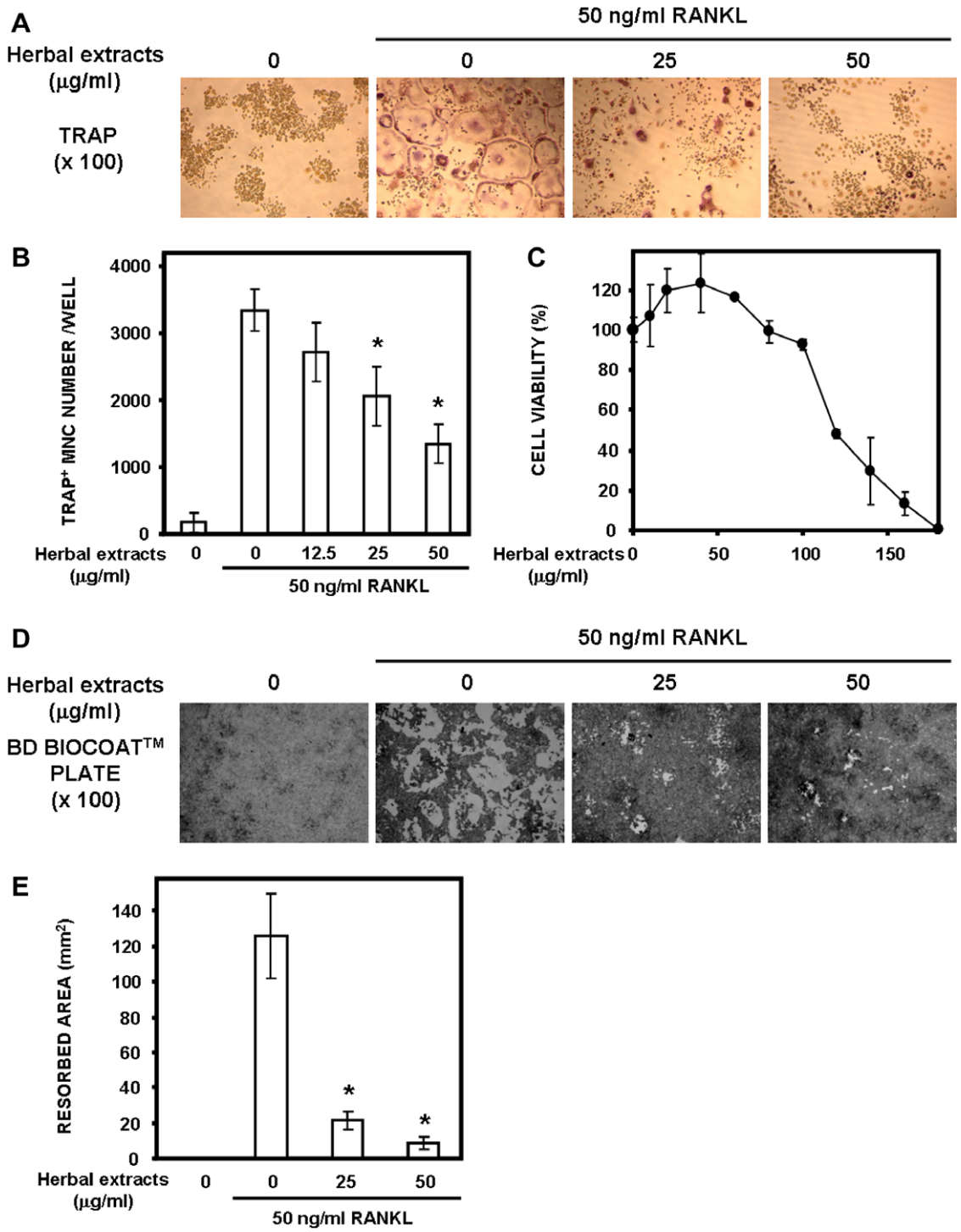

Fig. 1. Inhibition by *C. zeylanicum* extract of RANKL-induced osteoclastogenesis in RAW 264.7 cells. (A, B) Effects of *C. zeylanicum* extract on TRAP$^+$ MNCs formation. RAW 264.7 cells were cultured with RANKL in the presence or absence of *C. zeylanicum* extract (12.5–50 μg/ml). After 3 days of culturing, RANKL-treated cells were fixed and stained for TRAP. Graphical displays of cell morphology (A) and relative number of TRAP$^+$ MNCs (B). (C) Effects of *C. zeylanicum* extract on cell viability. Cells were cultured in the presence of various concentrations of *C. zeylanicum* extract for 48 h, after which viability was assessed by MTT assay. (D, E) Effects of *C. zeylanicum* extract on bone resorption by mature osteoclasts. RAW 264.7 cells on calcium phosphate apatite-coated plate (BioCoat™ Osteologic™ Bone Cell Culture System) were incubated with RANKL in the presence or absence of *C. zeylanicum* extract (25–50 μg/ml) for 8 days. Resorption pits (magnification, ×100) (D) and, total resorbed area (square millimeters) (E). The values represent means ± SD of triplicate determinations; $^*p < 0.01$ compared to control group.

MCA exceed those of CA. The other components exhibited no suppressive effects at high concentrations.

We also demonstrated that 2-MCA completely inhibits both TRAP$^+$ MNCs formation and bone resorption in RANKL-stimulated cells. These results indicate that CA and 2-MCA are the active components of *C. zeylanicum* extract.

3. Discussion and conclusions

Several recent reports indicate the potential role of certain herbs and their components in providing protection against osteoporosis, suggesting that consumption of herbs may contribute to bone remodeling.[13–17] Cinnamon bark or cortex is a popular natu-

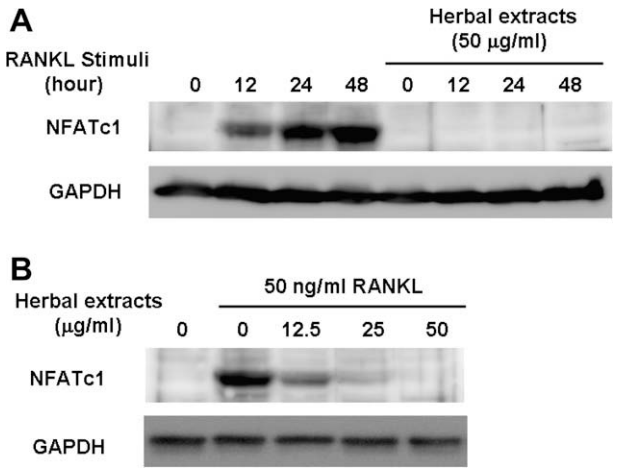

Fig. 2. The effects of *C. zeylanicum* extract on NFATc1 expression in osteoclastogenesis. (A) Time course analysis of NFATc1 expression. RAW 264.7 cells were cultured with RANKL in the presence or absence of *C. zeylanicum* extract (50 μg/ml) for the indicated times. The cell lysates were fractioned by 8% polyacrylamide gel electrophoresis and subjected to immunoblotting with an antibody for NFATc1 or GAPDH. (B) RAW 264.7 cells were cultured with RANKL with the indicated concentrations of *C. zeylanicum* extract for 48 h. NFATc1 expression was determined by immunoblotting with an anti-NFATc1 antibody.

ral spice and herb commonly used in traditional Chinese medicine, with numerous putative health benefits, but little is known about the effects of cinnamon bark on bone metabolism. Our study is the first to indicate that cinnamon bark extract, as *C. zeylanicum* extract, inhibits the RANKL-induced NFATc1 pathway in mouse RAWs and prevents osteoclastogenesis.

The RANKL-induced NFATc1 pathway plays an integral role in osteoclastogenesis and can be regarded as a promising target for therapeutic intervention in bone diseases. Urushibara et al. reported that inhibiting this pathway blocked osteoclast differentiation and bone destruction.[28] In our study, we initially used a screening approach to identify the effects of several herbal extracts on NFATc1-mediated signal transduction in a RAW264.7 murine cell line. We then discovered that *C. zeylanicum* extract suppressed to a remarkable degree the expression of NFATc1 in osteoclastogenesis and that the inhibitory effects of *C. zeylanicum* extract correlate with its modulation in $TRAP^+$ MNC formation, known to be an osteoclast-specific marker protein expressed in functionally mature osteoclasts. Thus, *C. zeylanicum* extract is likely to inhibit RANKL-induced osteoclast formation by selectively inhibiting NFATc1 expression. Several plant extracts and compounds have the capacity to prevent of bone loss at menopause by inducing osteoclast apoptosis.[29–31] This suggests that modulating osteoclast lifespan may be a key step in regulating bone resorption. However,

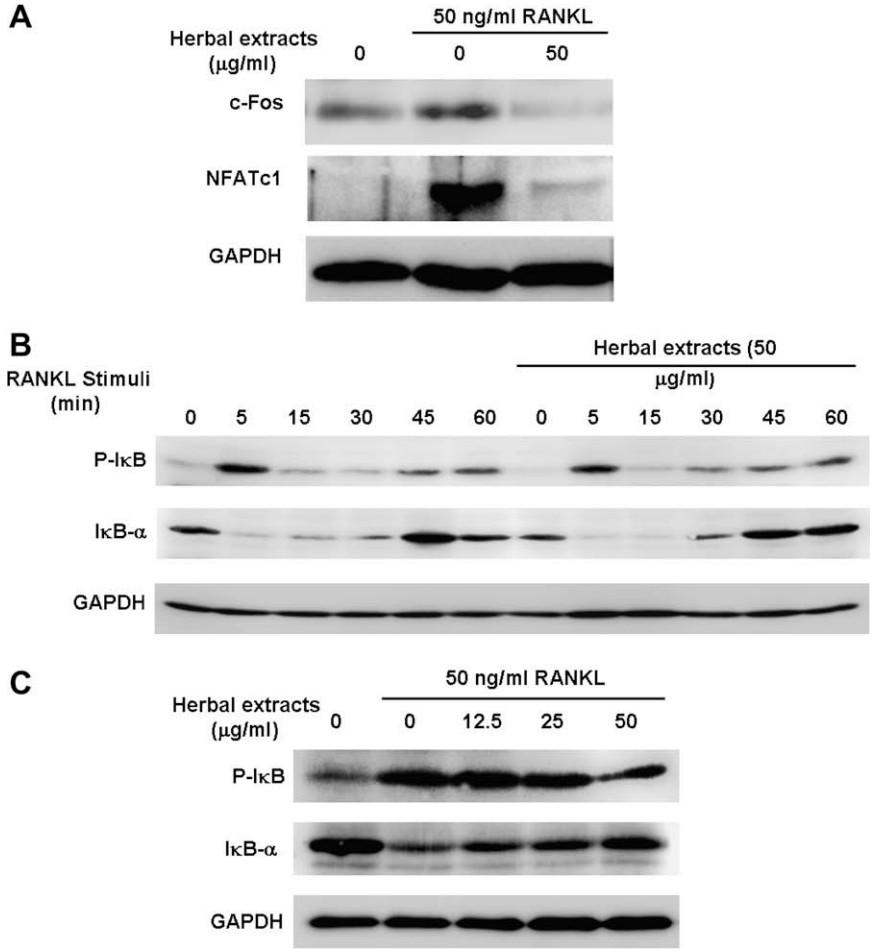

Fig. 3. Effects of *C. zeylanicum* extract on the expression of essential mediators of RANKL signaling. (A) Expression of c-*fos* and NFATc1 in RAWs stimulated in the presence or absence of *C. zeylanicum* extract. To evaluate the nuclear level of *c-fos*, western blot analysis conducted using nuclear extracts from untreated and *C. zeylanicum*-treated RAW 264.7 cells stimulated with RANKL for 30 min. To evaluate NFATc1 expression, RAW 264.7 cells were incubated with RANKL in the presence or absence of *C. zeylanicum* extract for 24 h. Cell lysates were fractioned by 8% polyacrylamide gel electrophoresis and immunoblotted with an antibody for c-*fos*, NFATc1, or GAPDH. (B) *C. zeylanicum* extract modestly inhibits RANKL-induced IκB-α phosphorylation and degradation. RAW 264.7 cells (1×10^6) were treated with RANKL (50 ng/ml) for the indicated times. Cytoplasmic extracts were prepared to check the following: levels of phosphorylated IκB-α (B, top); levels of IκB-α by Western blot analysis (B, middle). RAW 264.7 cells (1×10^6) were coincubated with either medium or *C. zeylanicum* extract (12.5–50 μg/ml) for 1 h and then treated with RANKL (50 ng/ml) for 10 min. Cytoplasmic extracts were prepared, fractionated on 8% SDS–PAGE, and electrotransferred to nitrocellulose membranes. Western blot analysis was done with anti-phosphorylated IκB-α (C, top) and anti-IκB-α (C, middle) antibodies.

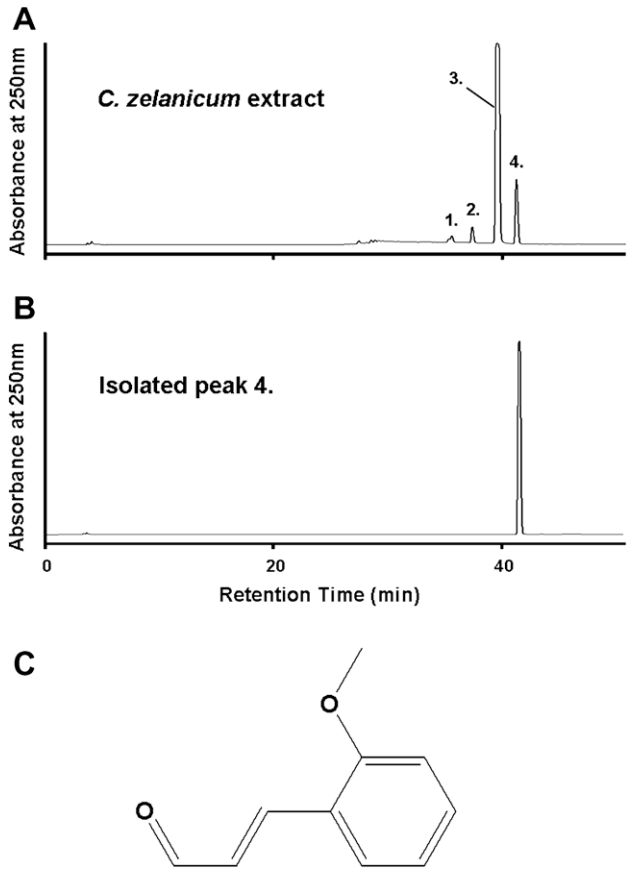

Fig. 4. HPLC chromatograms of *C. zeylanicum* extract. (A) HPLC profile of four components in *C. zeylanicum* extract. (1) cinnamyl alcohol, (2) cinnamic acid, (3) cinnamaldehyde, and (4) unknown. (B) HPLC analysis of isolated peak 4. (C) Structure of peak 4, 2-MCA. Peak 4 was purified by HPLC and its structure confirmed by NMR analysis.

the effects of *C. zeylanicum* extract may involve another mechanism, since *C. zeylanicum* extract-treatment cells were not observed to influence cell viability in either the absence or presence of RANKL-stimuli (data not shown).

We found that *C. zeylanicum* extract strongly suppressed expressed c-*fos*, which plays an exclusive role among the AP-1 proteins. Its signal pathway is known to be centrally involved in RANKL-induced NFATc1 expression. Putative AP-1 binding sites have been found in the promoter region of NFATc1, and osteoclast precursors lacking c-*fos* are free of NFATc1 expression. Our results raise the possibility that *C. zeylanicum* extract inhibits RANKL-induced osteoclast formation at the early stages of osteoclastogenesis in RAWs, inhibiting NFATc1 expression.

Suppressing NF-κB also affects NFATc1 expression. Takatsuna et al. have shown that NF-κB inhibitor (−)-dehydroxymethylepoxyquinomicin regulates NFATc1 expression and inhibits bone resorption.[32] NF-κB activation depends on two pathways[4]: The classic NF-κB pathway involves phosphorylations and ubiquitination-dependent degradation of IκBs; the alternative pathway is responsible for activating p52:RelB dimers. In a genomic study, p50/p52-deficient mice, not p50- or p52-deficient mice, exhibited bone abnormalities due to defects in osteoclast differentiation,[33,34] suggesting the function of cross-talk between the classic and alternative pathways. To explore the possibility that *C. zeylanicum* extract may inhibit NF-κB activation, we investigated the effects of *C. zeylanicum* extract on phosphorylation of IkB. We found that *C. zeylanicum* extract barely inhibits NF-κB activation of classic pathways at a dose of 12.5–50 μg/ml, which is insufficient for both NFATc1 expression and osteoclast formation. Thus, the inhibitory effects of *C. zeylanicum* extract on NFATc1 expression may be attributable primarily to the inhibition of c-*fos* expression, not NF-κB activation, since the inhibition of the classical pathway is an essential NF-κB inhibitor function. In this study, we demonstrated that *C. zeylanicum* extract also suppresses bone resorption, suggesting that *C. zeylanicum* extract may modulate the bone remodeling process by controlling bone resorption through inhibition of NFATc1 expression.

We found first that CA and 2-MCA may behave as inhibitors in the RANKL-induced NFATc1 pathway and of osteoclastogenesis. In particular, 2-MCA appears to strongly inhibit at low concentrations (1-2 μM), although the content of CA is higher than that of 2-MCA in *C. zeylanicum* extract (Fig. 4A). Kim et al. report that CA and 2-MCA have been identified as NF-κB inhibitors from *C. cassia* using LPS-stimulated RAW 264.7 cells and that these compounds show IC_{50} values of 43 and 31 μM, respectively.[35] However, the only NF-κB inhibitor function of *C. zeylanicum* extract has difficulty in accounting for the inhibitory effects of *C. zeylanicum* extract on osteoclastogenesis. This is because *C. zeylanicum* extract inhibits NF-κB activation weakly at doses of 12.5–50 μg/ml, despite a marked osteoclastic inhibition at the same concentrations. In addition, we observed virtually no inhibitory effects of 2-MCA on NF-κB activation at concentrations as low as 1–2 μM in the RANKL-stimulated RAWs model (Supplementary data). Thus, to understand the inhibitory effects of these compounds on osteoclastogenesis, we have also been compelled to focus on other essential factors, including *c-fos*. In contrast, we found that cinnamic acid and cinnamyl alcohol fail to inhibit $TRAP^+$ MNCs formation in RANKL-stimulated RAWs (data not shown), implying that the aldehyde functional groups of CA and 2-MCA may be essential for the inhibition of osteoclastogenesis through the suppression of NFATc1 expression. In addition, our findings raise the possibility that the derivatives of benzene ring in CA, including 2-MCA, might enhance the potent inhibitory effects on osteoclastogenesis. We have demonstrated that the inhibitory action of 2-MCA would be more potent than that of CA, a conclusion in agreement with several reports that have shown varying inhibitory effects between CA and the derivatives of its benzene ring with respect to antimicrobial activity and effectiveness as NF-κB inhibitors.[35,36]

Takayanagi et al. have shown that administering FK506, a calcineurin inhibitor, induces bone mass reductions despite a blockade of osteoclast differentiation.[37] The modulation of NFAT proteins likely affects the normal homeostasis of the skeletal system, since NFAT cooperates with Osterix, an essential osteogenic factor, and accelerates osteoblast differentiation and bone formation. However, recent reports indicate that extract of the bark of *C. cassia* blume, a species related to *C. zeylanicum*, has estrogenic effects and may accelerate bone formation, indicating that the complex mechanisms involved in the regulation of NFATc1 expression may evoke homeostasis of the skeletal system.[22] Additional study is needed to understand the molecular events involved, including evaluations of potential active components having estrogenic effects.

In summary, our study clearly demonstrates that cinnamon bark strongly inhibits osteoclastic activity. The mechanism of action involves the suppression of NFATc1-mediated signal transduction. We also identified CA and 2-MCA as its active components. The results of this study suggest that *C. zeylanicum* extract may be highly effective in the treatment of pathological bone disruption-related diseases, including osteoporosis, bone metastasis, and rheumatoid arthritis.

4. Materials and methods

4.1. Materials

All media components were purchased from Invitrogen (Carlsbad, CA). Antibodies against NFATc1 and nuclear inhibitory κB-α

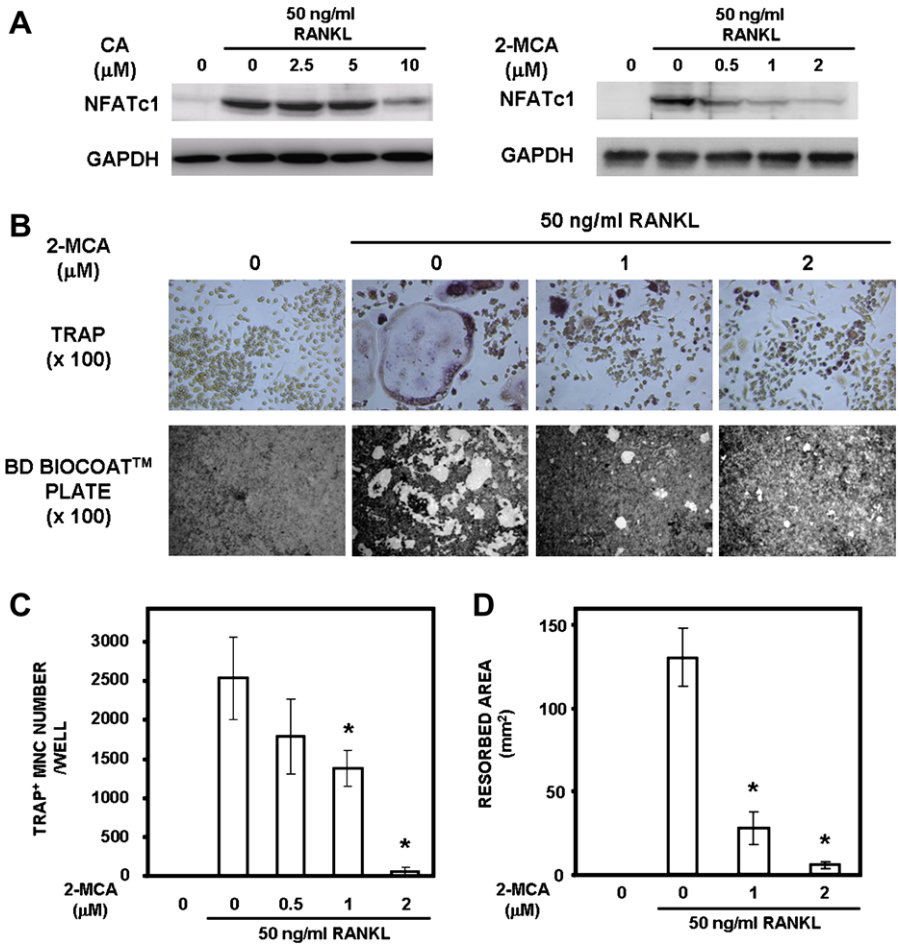

Fig. 5. Effects of active components including *C. zeylanicum* extract on RANKL-induced osteoclastogenesis in RAW 264.7 cells. (A) RAW 264.7 cells were cultured with RANKL along with the indicated concentrations of CA or 2-MCA for 48 h. NFATc1 expression was determined by immunoblotting with an antibody for NFATc1 or GAPDH. (B top and C) Effects of 2-MCA on TRAP$^+$ MNCs formation. RAW 264.7 cells were cultured with RANKL in the presence or absence of 2-MCA (0.5–2 μM). After 3 days of culturing, RANKL-treated cells were fixed and stained for TRAP. Graphical displays of cell morphology (B top) and relative number of TRAP$^+$ MNCs (C). (B bottom and D) Effects of 2-MCA on bone resorption by mature osteoclasts; RAW 264.7 cells on calcium phosphate apatite-coated plate (BioCoat^{TM} Osteologic^{TM} Bone Cell Culture System) were incubated with RANKL in the presence or absence of *C. zeylanicum* extract (25–50 μg/ml) for 8 days. Resorption pits (magnification × 100) (B bottom) and, total resorbed area (square millimeters) (D). The values represent means ± SD of triplicate determinations; $^*p < 0.01$ compared to control group.

(IκB-α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal antibody to c-*fos* was obtained from Abcam, Ltd. (Cambridgeshire, UK). Antibodies against GAPDH were purchased from Ambion, Inc. (Austin, TX). Antibodies against phospho-IkBα were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA); goat anti-mouse horseradish peroxidase and BioCoat^{TM} Osteologic^{TM} Bone Cell Culture System from BD Biosciences (San Jose, CA); and CA, 2-MCA, cinnamyl alcohol, cinnamic acid, and MTT from Sigma–Aldrich (St. Louis, MO). RANKL was purchased from Pepro Tech EC, Ltd. (London, UK). The sample of *C. zeylanicum* was prepared from the bark of *C. zeylanicum* Blume purchased commercially (Mimuro, Inc., Shizuoka, Japan) and pulverized. *C. zeylanicum* (100 mg) extract was obtained by extraction with 1 ml of 99.5% ethanol at room temperature for 3 days. The resultant solution was centrifuged and the supernatant removed, after which the solution was evaporated to dryness under vacuum conditions. The residue was dissolved in dimethylsulfoxide.

4.2. Cell cultures

The RAW264.7 mouse macrophage cell line was obtained from American Type Culture Collection (Manassas, VA). RAW264.7 cells were maintained in DMEM medium supplemented with 10% FBS and 2 mM glutamine, and antibiotics. Incubation was performed at 37 °C in 5% CO_2 in humidified air. For osteoclast generation and all other experiments, α-MEM medium was used.

4.3. TRAP staining

After 3 days of culturing, RAW 264.7 cells treated with RANKL were fixed with a solution containing acetone and citrate for 30 s and washed twice with double-distilled water. TRAP staining was performed using a kit purchased from Sigma according to the manufacturer's instructions. TRAP$^+$ cells with four or more nuclei were scored as TRAP$^+$ MNCs under light microscopy. For statistical evaluations, cell counting was done using four different fields of view and was repeated four times.

4.4. Resorption pit assay

This assay was performed according to the manufacturer's instructions. Briefly, to determine the effects of cinnamon bark extract (or its active components) on pit formation, RAW cells were pretreated with cinnamon bark extract (or active components) for an hour, before adding RANKL (50 ng/ml) to calcium phosphate apatite-coated plates (BioCoat^{TM} Osteologic^{TM} Bone Cell Culture

System) in the culture medium, used in most experiments to quantify osteoclastic cell-mediated mineral resorption. Mammalian osteoclastic cells have been shown to behave on these thin films much like they do on other bone or dentine slices.[38] After 8 days of incubation, the plates were treated with 5% sodium hypochlorite for 5 min. The plates were washed with water, then dried and photographed.

4.5. Western blot

RAWs were lysed with lysis buffer (10 mM HEPES [pH 7.8], 150 mM NaCl, 2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and protease inhibitors). Each extract was fractionated on a polyacrylamide–SDS gel, after which proteins were transferred to nitrocellulose membranes using a semidry blotter (BIO CRAFT Co., Ltd., Tokyo, Japan) and incubated in blocking solution (5% non-fat dry milk in PBS containing 0.1% Tween-20) for 1 h to reduce non-specific binding. Membranes were then exposed to primary antibodies (overnight at 4 °C). Thereafter, the blot was washed, exposed to HRP-conjugated secondary Abs for 1 h, and finally detected by ECL Plus Western Blotting Detection System (GE Healthcare Biosciences, Piscataway, NJ).

4.6. HPLC

HPLC employed a JASCO Corp. gradient system equipped with dual Model PU-2089 plus pumps (10 mL pump heads), a Rheodyne (Cotati, CA) Model 7725i equipped with a 5 ml sample loop, and a JASCO Corp. Model MD-2010 plus multi-UV detector. Two mobile phase solvents were employed. Solvent A was prepared by adding concentrated acetic acid (0.1%) to deionized water. Solvent B was prepared by adding acetic acid (0.1%) to HPLC grade acetonitrile. An analytical method employed a reverse phase column (Develosil C_{18}, 5μm, 4.6 × 250 mm, Nomura Chemical Corp., Ltd.) and the following mobile gradient: 0–15 min, linear gradient from 99% solvent A to 90% solvent A; 15–50 min, linear gradient to 100% solvent B. The flow rate was constant at 1.0 ml min^{-1}. For isolation, HPLC employed a reverse phase column (Develosil C_{18}, 5 μm, 20 × 250 mm, Nomura Chemical Corp., Ltd., Aichi, Japan) under the same chromatographic condition. The flow rate was constant at 2.0 ml min^{-1}.

4.7. trans-2-methoxycinnamaldehyde

2-MCA was isolated and purified from the bark of *C. zeylanicum* by HPLC. The solution eluted under the chromatographic peak was collected and immediately frozen at −80 °C (dry ice bath). The resulting eluents were freeze-dried to render a solid, dry product. The structure of purified 2-MCA was confirmed by ^{1}H NMR and two-dimensional correlated spectroscopy experiments (JNM-AL 400 NMR spectrometer system, a JEOL, Ltd., Tokyo, Japan) and determined by comparison against spectra for authentic 2-MCA. ^{1}H NMR (400 MHz, dimethylsulfoxide-d_6) spectrum of *trans*-2-methoxycinnamaldehyde was assigned as follows: δ 3.88 (s, 1H), 6.84 (dd, 1H, J = 16.0, 7.6 Hz), 7.01 (t, 1H, J = 7.6, 7.6 Hz), 7.12 (d, 1H, J = 8.4 Hz), 7.47 (m, 1H, J = 8.0, 7.9, 1.6 Hz), 7.73 (dd, 1H, J = 7.8, 1.6 Hz), 7.89 (d, 1H, J = 16.4 Hz), 9.65 (d, 1H, J = 7.6 Hz). ^{1}H NMR (400 MHz, dimethylsulfoxide-d_6) spectrum of authentic *trans*-2-methoxycinnamaldehyde was assigned as follows: δ 3.89 (s, 1H), 6.82 (dd, 1H, J = 16.0, 8.0 Hz), 6.99 (t, 1H, J = 7.6, 7.6 Hz), 7.07 (d, 1H, J = 8.4 Hz), 7.43 (m, 1H, J = 8.4, 7.9, 1.6 Hz), 7.68 (dd, 1H, J = 7.6, 1.6 Hz), 7.85 (d, 1H, J = 16.0 Hz), 9.63 (d, 1H, J = 7.6 Hz). The UV-spectrum appeared as separable peaks with λ_{max} at 232, 288, and 337 nm, respectively.

4.8. Statistical analysis

All data are expressed as means ± SD of triplicate determination. Statistical analyses were performed by ANOVA with Dunnett's multiple comparison of mean test.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.09.036.

References and notes

1. Manolagas, S. C. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr. Rev.* **2000**, *21*, 115.
2. Rodan, G. A.; Martin, T. J. Therapeutic approaches to bone diseases. *Science* **2000**, *289*, 1508.
3. Suda, T.; Takahashi, N.; Martin, T. J. Modulation of osteoclast differentiation. *Endocr. Rev.* **1992**, *13*, 66.
4. Asagiri, M.; Takayanagi, H. The molecular understanding of osteoclast differentiation. *Bone* **2007**, *40*, 251.
5. Li, J.; Sarosi, I.; Yan, X. Q.; Morony, S.; Capparelli, C.; Tan, H. L.; McCabe, S.; Elliott, R.; Scully, S.; Van, G.; Kaufman, S.; Juan, S. C.; Sun, Y.; Tarpley, J.; Martin, L.; Christensen, K.; McCabe, J.; Kostenuik, P.; Hsu, H.; Fletcher, F.; Dunstan, C. R.; Lacey, D. L.; Boyle, W. J. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl. Acad. Sci. U S A.* **2000**, *97*, 1566.
6. Dougall, W. C.; Glaccum, M.; Charrier, K.; Rohrbach, K.; Brasel, K.; De Smedt, T.; Daro, E.; Smith, J.; Tometsko, M. E.; Maliszewski, C. R.; Armstrong, A.; Shen, V.; Bain, S.; Cosman, D.; Anderson, D.; Morrissey, P. J.; Peschon, J. J.; Schuh, J. RANK is essential for osteoclast and lymph node development. *Genes Dev.* **1999**, *13*, 2412.
7. Wong, B. R.; Josien, R.; Lee, S. Y.; Vologodskaia, M.; Steinman, R. M.; Choi, Y. The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. *J. Biol. Chem.* **1998**, *273*, 28355.
8. Galibert, L.; Tometsko, M. E.; Anderson, D. M.; Cosman, D.; Dougall, W. C. The involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily. *J. Biol. Chem.* **1998**, *273*, 34120.
9. Matsumoto, M.; Sudo, T.; Saito, T.; Osada, H.; Tsujimoto, M. Involvement of p38 mitogen-activated protein kinase signaling pathway in osteoclastogenesis mediated by receptor activator of NF-kappa B ligand (RANKL). *J. Biol. Chem.* **2000**, *275*, 31155.
10. Miyazaki, T.; Katagiri, H.; Kanegae, Y.; Takayanagi, H.; Sawada, Y.; Yamamoto, A.; Pando, M. P.; Asano, T.; Verma, I. M.; Oda, H.; Nakamura, K.; Tanaka, S. Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. *J. Cell Biol.* **2000**, *148*, 333.
11. Johnson, R. S.; Spiegelman, B. M.; Papaioannou, V. Pleiotropic effects of a null mutation in the c-fos proto-oncogene. *Cell* **1992**, *71*, 577.
12. Takayanagi, H.; Kim, S.; Koga, T.; Nishina, H.; Isshiki, M.; Yoshida, H.; Saiura, A.; Isobe, M.; Yokochi, T.; Inoue, J.; Wagner, E. F.; Mak, T. W.; Kodama, T.; Taniguchi, T. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell* **2002**, *3*, 889.
13. Mühlbauer, R. C.; Li, F. Effect of vegetables on bone metabolism. *Nature* **1999**, *401*, 343.
14. Park, C. K.; Lee, Y.; Chang, E. J.; Lee, M. H.; Yoon, J. H.; Ryu, J. H.; Kim, H. H. Bavachalcone inhibits osteoclast differentiation through suppression of NFATc1 induction by RANKL. *Biochem. Pharmacol.* **2008**, *75*, 2175.
15. Yu, S.; Chen, K.; Li, S.; Zhang, K. In vitro and in vivo studies of the effect of a Chinese herb medicine on osteoclastic bone resorption. *Chin. J. Dent. Res.* **1999**, *2*, 7.
16. Kim, C.; Ha, H.; Lee, J. H.; Kim, J. S.; Song, K.; Park, S. W. Herbal extract prevents bone loss in ovariectomized rats. *Arch. Pharm. Res.* **2003**, *26*, 917.
17. Dong, G. C.; Chen, H. M.; Yao, C. H. A novel bone substitute composite composed of tricalcium phosphate gelatin and drynaria fortunei herbal extract. *J. Biomed. Mater. Res. A* **2008**, *84*, 167.
18. Nir, Y.; Potasman, I.; Stermer, E.; Tabak, M.; Neeman, I. Controlled trial of the effect of cinnamon extract on *Helicobacter pylori*. *Helicobacter* **2000**, *5*, 94.
19. Tanaka, S.; Yoon, Y. H.; Fukui, H.; Tabata, M.; Akira, T.; Okano, K.; Iwai, M.; Iga, Y.; Yokoyama, K. Antiulcerogenic compounds isolated from Chinese cinnamon. *Planta Med.* **1989**, *55*, 245.
20. Atta, A. H.; Alkofahi, A. Anti-nociceptive and anti-inflammatory effects of some Jordanian medicinal plant extracts. *J. Ethnopharmacol.* **1998**, *60*, 117.
21. Dugoua, J. J.; Seely, D.; Perri, D.; Cooley, K.; Forelli, T.; Mills, E.; Koren, G. From type 2 diabetes to antioxidant activity: a systematic review of the safety and efficacy of common and cassia cinnamon bark. *Can. J. Physiol. Pharmacol.* **2007**, *85*, 837.
22. Lee, K. H.; Choi, E. M. Stimulatory effects of extract prepared from the bark of Cinnamomum cassia blume on the function of osteoblastic MC3T3-E1 cells. *Phytother. Res.* **2006**, *20*, 952.

23. Yang, J.; Chen, L. H.; Zhang, Q.; Lai, M. X.; Wang, Q. Quality assessment of cortex cinnamomi by HPLC chemical fingerprint principle component analysis and cluster analysis. *J. Sep. Sci.* **2007**, *30*, 1276.
24. He, Z. D.; Qiao, C. F.; Han, Q. B.; Cheng, C. L.; Xu, H. X.; Jiang, R. W.; But, P. P.; Shaw, P. C. Authentication and quantitative analysis on the chemical profile of cassia bark (cortex cinnamomi) by high-pressure liquid chromatography. *J. Agric. Food Chem.* **2005**, *53*, 2424.
25. Morozumi, S. Isolation purification and antibiotic activity of *o*-methoxycinnamaldehyde from cinnamon. *Appl. Environ. Microbial.* **1978**, *36*, 577.
26. Montes-Belmont, R.; Carvajal, M. Control of *Aspergillus flavus* in maize with plant essential oils and their components. *J. Food Prot.* **1998**, *61*, 616.
27. Hasegawa, A.; Yoshino, M.; Nakamura, H.; Ishii, I.; Watanabe, T.; Kiuchi, M.; Ishikawa, T.; Ohmori, S.; Kitada, M. Identification of inhibitory component in cinnamon-*O*-methoxycinnamaldehyde inhibits CYP1A2 and CYP2E1. *Drug Metab. Pharmacokinet.* **2002**, *17*, 229.
28. Urushibara, M.; Takayanagi, H.; Koga, T.; Kim, S.; Isobe, M.; Morishita, Y.; Nakagawa, T.; Löeffler, M.; Kodama, T.; Kurosawa, H.; Taniguchi, T. The antirheumatic drug leflunomide inhibits osteoclastogenesis by interfering with receptor activator of NF-kappa B ligand-stimulated induction of nuclear factor of activated T cells c1. *Arthritis Rheum.* **2004**, *50*, 794.
29. García Palacios, V.; Robinson, L. J.; Borysenko, C. W.; Lehmann, T.; Kalla, S. E.; Blair, H. C. Negative regulation of RANKL-induced osteoclastic differentiation in RAW264.7 Cells by estrogen and phytoestrogens. *J. Biol. Chem.* **2005**, *280*, 13720.
30. Wattel, A.; Kamel, S.; Mentaverri, R.; Lorget, F.; Prouillet, C.; Petit, J. P.; Fardelonne, P.; Brazier, M. Potent inhibitory effect of naturally occurring flavonoids quercetin and kaempferol on in vitro osteoclastic bone resorption. *Biochem. Pharmacol.* **2003**, *65*, 35.
31. Boissy, P.; Andersen, T. L.; Abdallah, B. M.; Kassem, M.; Plesner, T.; Delaissé, J. M. Resveratrol inhibits myeloma cell growth, prevents osteoclast formation and promotes osteoblast differentiation. *Cancer Res.* **2005**, *65*, 9943.
32. Takatsuna, H.; Asagiri, M.; Kubota, T.; Oka, K.; Osada, T.; Sugiyama, C.; Saito, H.; Aoki, K.; Ohya, K.; Takayanagi, H.; Umezawa, K. Inhibition of RANKL-induced osteoclastogenesis by (−)-DHMEQ, a novel NF-kappaB inhibitor, through downregulation of NFATc1. *J. Bone Miner. Res.* **2005**, *20*, 653.
33. Franzoso, G.; Carlson, L.; Xing, L.; Poljak, L.; Shores, E. W.; Brown, K. D.; Leonardi, A.; Tran, T.; Boyce, B. F.; Siebenlist, U. Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev.* **1997**, *11*, 3482.
34. Iotsova, V.; Caamaño, J.; Loy, J.; Yang, Y.; Lewin, A.; Bravo, R. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat. Med.* **1997**, *3*, 1285.
35. Reddy, A. M.; Seo, J. H.; Ryu, S. Y.; Kim, Y. S.; Kim, Y. S.; Min, K. R.; Kim, Y. Cinnamaldehyde and 2-methoxycinnamaldehyde as NF-kappaB inhibitors from *Cinnamomum cassia*. *Planta Med.* **2004**, *70*, 823.
36. Quale, J. M.; Landman, D.; Zaman, M. M.; Burney, S.; Sathe, S. S. In vitro activity of *Cinnamomum zeylanicum* against azole resistant and sensitive Candida species and a pilot study of cinnamon for oral candidiasis. *Am. J. Chin. Med.* **1996**, *24*, 103.
37. Koga, T.; Matsui, Y.; Asagiri, M.; Kodama, T.; de Crombrugghe, B.; Nakashima, K.; Takayanagi, H. NFAT and Osterix cooperatively regulate bone formation. *Nat. Med.* **2005**, *11*, 880.
38. Loomer, P. M.; Ellen, R. P.; Tenenbaum, H. C. Osteogenic and osteoclastic cell interaction: development of a co-culture system. *Cell Tissue Res.* **1998**, *294*, 99.