Castalagin exerts inhibitory effects on osteoclastogenesis through blocking a broad range of signaling pathways with low cytotoxicity

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Abbreviations: OCLs, osteoclasts; RANKL, receptor activator of nuclear factor kappa-B ligand; BMMs, bone marrow-derived macrophages; MAPKs, mitogen-activated protein kinases; Erk, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; NFATc1, nuclear factor of activated T cells cytoplasmic-1; HO-1, heme oxygenase-1; M-CSF, macrophage colony-stimulating factor; RANK, receptor activator of nuclear factor kappa-B; NF-κB, nuclear factor kappa B; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; TRAF 6, TNF receptor associated factor; Rac 1, Ras-related C3 botulinum toxin substrate; Nox 1, NADPH oxidase 1; 1 α-MEM, α-minimal essential medium; TRAP, tartrate-resistant acid phosphatase; NAC, *N*-acetylcysteine; IκBα, inhibitor of nuclear factor kappa B alpha

Abstract

Castalagin is a rare plant polyphenol that is classified as a hydrolyzable tannin. Although it has anti-oxidant, anti-tumorigenic, and leishmanicidal effects, the utility of castalagin against bone diseases remain to be elucidated. Here, we investigated the effects of castalagin on the differentiation of osteoclasts (OCLs), multinucleated bone resorbing cells. After stimulation with receptor activator of nuclear factor kappa-B ligand (RANKL), the formation of OCLs from bone marrow-derived macrophages (BMMs) was significantly inhibited by castalagin even at 1 μM. However, castalagin displayed little cytotoxicity at a higher concentration of 50 μM. The effects of castalagin on intracellular signaling during OCL differentiation showed that castalagin suppresses RANKL-stimulated phosphorylation of major signaling pathways including, Akt, extracellular signal-regulated kinase (Erk), Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinases (MAPKs), and inhibitor of nuclear factor kappa B alpha (IκB). Moreover, following castalagin treatment, the protein levels of nuclear factor of activated T cells cytoplasmic-1 (NFATc1), a master regulator for OCL differentiation, and NF-κB were decreased. Thus, castalagin exerts inhibitory effects on

osteoclastogenesis through blockage of a broad range of signaling pathways, but has low cytotoxicity.

Introduction

Castalagin is a rare plant polyphenol that is mainly distributed in oak and chestnut wood, and in the stem bark of *Anogeissus leicarpus* and *Terminalia Avicennoides* (Hager *et al.*, 2008). Castalagin is part of a particular group of ellagitannins that are composed of a series of highly hydrosoluble C-glucosidic variants (Vivas *et al.*, 2004). Concerning the bioactivities of castalagin, only a few studies have been reported. A previous study using *in vitro* systems has reported that castalagin displays a powerful leishmanicidal activity compared with other ellagitanins (Shuaibu *et al.*, 2008). Moreover, castalagin has been shown to have anti-cancer effects *in vitro*. Fridrich *et al*. (Fridrich *et al.*, 2008) have reported that castalagin suppresses phosphorylation of the epidermal growth factor receptor in human colon carcinoma HT29 cells. However, beyond this, whether castalagin has pharmacological effects useful in the prevention of bone diseases such as osteoporosis, periodontitis and rheumatoid arthritis remains unknown. Using an *in vitro* system, we show here that castalagin has a powerful

inhibitory effect on the differentiation of osteoclasts (OCLs) and their bone resorbing activity with an *in vitro* system.

OCLs are bone resorbing multinucleated cells that are derived from monocyte/macrophage precursor cells (Teitelbaum, 2000). Their differentiation pathway from the precursor cells into OCLs is responsible for the 2 essential cytokines: receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). When RANKL binds to the receptor, receptor activator of nuclear factor kappa-B (RANK), the key signaling pathways in OCL differentiation is activated: nuclear factor of activated T cells cytoplasmic-1 (NFATc1), nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K)/Akt, Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (Erk), p38 mitogen-activated protein kinases (MAPKs) (Darnay *et al*., 1999; Matsumoto *et al.*, 2000; Zhang *et al.*, 2001). In addition to these signaling pathways, reactive oxygen species (ROS) have been shown to be essential for the OCL differentiation (Ha *et al.*, 2004; Lee *et al.*, 2005). The transiently increased ROS triggers a signaling cascade involving TRAF 6, Rac 1, and NADPH oxidase 1 (Nox 1). In contrast, blocking ROS

by addition of the reducing agent *N*-acetylcysteine or diphenylene iodonium, an inhibitor of Nox, completely inhibits the osteoclastogenesis via various signaling pathways, such as JNK, p38 MAPKs, and Erk (Lee *et al.*, 2005). Recently, we demonstrated that the expression of the anti-oxidant enzyme heme-oxigenase 1 (HO-1) by pharmacological or genetic induction clearly inhibits OCL differentiation ((Sakai et al., 2011; Sakai et al., 2012) . In this study, we report the inhibitory mechanisms of castalagin on OCL differentiation using *in vitro* OCL culture systems.

Materials and methods

Reagents

Human M-CSF was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Recombinant human soluble RANKL was prepared as described previously (Hu *et al.*, 2008). Antibodies were purchased as follows: β-actin (Sigma-Aldrich, St. Louis, MO, USA), Src (Upstate Biotechnology, Lake Placid, NY, USA); anti-c-fms, anti-RANK, , anti-cFos, and anti-NFATc1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Abs specific for NF-κB, Erk1/2, phospho-Erk1/2, Akt, phospho-Akt,

JNK, phospho-JNK, p38 MAPK, phospho-p38 MAPK, and inhibitor of nuclear factor kappa B alpha (IκBα), phospho-IκBα were purchased from Cell Signaling Technology (Danvers, MA, USA);and cathepsin K antibody was prepared as described previously (Kamiya *et al.*, 1998). The Osteo Assay Plate was purchased from Corning (Corning, NY, USA). All other reagents, including phenylmethylsulfonyl fluoride and the protease inhibitor cocktail, were obtained from Sigma-Aldrich.

Isolation of castalagin

Isolation of castalagin was performed as described previously (Shuaibu *et al.*, 2008). Briefly, castalagin was isolated from the wood of *Castanea crenata*, an aqueous acetone extract of the dried bark was separated by Sephadex LH-20 column chromatography with H_2O containing increasing proportions of MeOH and finally with H_2O-acetone (1:1, v/v). The fractions containing castalagin were collected and further separated by Diaion HP20SS chromatography with 10-20% MeOH. Castalagin was obtained as a white crystalline powder from H_2O and identified by ^1H NMR spectroscopic comparison with an authentic sample. The structure of castalagin is shown in Fig.1.

Cell culture

Five-week-old male BALB/c mice were obtained from CLEA Japan, Inc. (Tokyo, Japan), and handled in our facilities under protocols approved by the Nagasaki University Animal Care Committee. Bone marrow-derived macrophages (BMMs) were isolated as described previously (Sakai et al., 2012) The BMMs were replated in culture plates and incubated in α-minimal essential medium (α-MEM) containing 10% FBS with 100 U/mL of penicillin and 100 μg/mL of streptomycin in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 72 h until the cells differentiated into multinucleated mature OCLs.

The cells were fixed with 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) activity using a previously described method (Hotokezaka *et al.*, 2002). TRAP-positive red-colored cells with 3 or more nuclei were considered mature OCLs. For bone resorption pit formation, BMMs were seeded onto Osteo Assay Plates coated with thin calcium phosphate films (Corning, NY, USA) and incubated with M-CSF and RANKL for 5 days until the mature OCLs resorbed the calcium

phosphate film. Cells were dissolved in 5% sodium hypochlorite. Images of the resorption pit were taken with a reverse phase microscope (Olympus, Tokyo, Japan). The ratios of the resorbed areas to the total areas were calculated using Image J image-analysis software (http://rsbweb.nih.gov/ij/) as described previously (Narahara *et al*., 2012).

Cell viability assay

Cells seeded in 96-well cell culture plates were incubated with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) for 1 h, and then the absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark^{TM}, Hercules, CA, USA). Castalagin was added the medium at the beginning.

Western blot analysis

BMMs were stimulated with or without RANKL in the presence of M-CSF for the indicated time. Cells were rinsed twice with ice-cold PBS, and lysed in a cell lysis buffer (50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1%

SDS, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail). The protein concentration of each sample was measured with BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). An equal amount of protein (5 μg) was applied to each lane. After SDS-PAGE, proteins were electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked with 3% milk in 5% BSA/TBST for 1 h at room temperature, probed with various antibodies overnight at 4 °C, washed, incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, and Dako, Glostrup, Denmark), and finally detected with ECL-Plus (GE Healthcare Life Sciences, Tokyo, Japan). The immunoreactive bands were analyzed by LAS1000 (Fuji Photo Film, Tokyo, Japan).

Immunofluorescence microscopy

BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 72 h in the presence of 0-50 μM castalagin. The cells were fixed with 4% paraformaldehyde at 4 °C for 30 min, permealized with 0.1% Triton X-100 for 10 min, and then blocked with 0.2% gelatin. For NF-κB localization, anti-p65 antibody was diluted 1:100 in 0.2% gelatin and incubated overnight at 4 °C, followed by incubation for 2 h at room

temperature with Alexa 488 conjugated anti-mouse immunoglobulin diluted in 1:100 in 0.2% gelatin. Microscope images of fluorescence were digitized using Carl Zeiss LSM 710 confocal microscope.

Reverse transcription and real-time quantitative PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using an oligo$(dT)_{15}$ primer (Promega, Madison, WI, USA) and Revertra Ace (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed using a MX3005P QPCR system (Agilent Technology, La Jolla, CA, USA). The cDNA was amplified using Brilliant III Ultra-Fast SYBR QPCR Master Mix (Agilent Technology, La Jolla, CA, USA) according to the manufacturer's instructions. The following primer sets were used:

β-actin:

5'-ACCCAGATCATGTTTGAGAC-3' forward and

5'-GTCAGGATCTTCATGAGGTAGT-3' reverse,

HO-1:

5'-CACGCATATACCCGCTACCT-3' forward and

5'-CCAGAGTGTTCATTCGAGCA-3' reverse,

Statistical analysis

All values are expressed as the means ± standard deviations for 3 independent experiments. Tukey-Kramer method was used to identify differences between concentrations when ANOVA indicated a significant difference (*$P < 0.05$ or **$P < 0.01$). Alternatively, 2-factor ANOVA was used.

Results

Castalagin inhibits osteoclastogenesis in vitro

To evaluate whether castalagin inhibits osteoclastogenesis, we first examined its effects on OCL differentiation from native BMMs treated with M-CSF (30 ng/mL) and RANKL (50 ng/mL). As shown in Fig 2A, TRAP activity staining showed that castalagin inhibited the formation of mononuclear and multinuclear OCLs. The number

of TRAP-positive, multinucleated OCLs decreased after castalagin treatment even at the lower concentration of 1μM (Fig. 2B). However, the cell viability was still maintained even at the higher concentrations of 25 and 50 μM (Fig. 2C). These results indicate that castalagin strongly inhibits osteoclastogenesis *in vitro* in the RANKL-induced culture system at low concentrations, but has scarcely any cytotoxicity, even at higher concentrations.

Effects of castalagin on the bone resorbing activity of OCLs

To further examine whether castalagin prevents the bone resorbing activity of OCLs, we performed a pit formation assay with BMM-derived OCLs after treatment with M-CSF and RANKL. As shown in Fig. 3A, castalagin significantly inhibited bone resorbing activity. When treated with 1μM castalagin, the bone resorbing activity was markedly reduced (Fig. 3A). The calculated resorption area of castalagin-treated OCLs was decreased compared with that of untreated OCLs (Fig. 3B). Thus, castalagin inhibits the physiological bone resorbing activity of OCLs.

Effects of castalagin on intracellular signaling and expression levels of OCL marker proteins

We further investigated the effects of castalagin on RANKL-induced intracellular signaling during the OCL differentiation from BMMs. Since OCL differentiation is known to be mainly regulated by several signaling pathways, including those modulated by NFATc1, NF-κB, PI3K/Akt, JNK, Erk, and p38 MAPKs (Boyle *et al.*, 2003). Therefore, we examined the effects of castalagin on phosphorylation of Akt, JNK, IκBα, Erk, and p38 MAPKs by western blotting. When BMMs were pre-incubated without or with 10 μM of castalagin for 12 h, and then further incubated for the indicated times (5, 10, 15, and 30 min) after stimulation with RANKL. As shown in Fig. 4, castalagin sufficiently blocked phosphorylation of Akt, Erk, and p38. In addition, castalagin moderately inhibited the phosphorylation of JNK and IκBα (Fig. 4). These results indicate that castalagin interferes with all the 5 signaling pathways such as Akt, Erk, JNK, p38 MAPKs and IκBα-dependent pathways.

To further analyze the effects of castalagin on osteoclastogenesis, we determined the expression levels of several OCL marker proteins by western blotting. RANK is a

RANKL receptor, while c-fms is an M-CSF receptor. NFATc1, NFκB, and c-fos are essential transcriptional factors for OCL differentiation, whereas c-Src is a non-receptor-type tyrosine kinase. Cathepsin K is the OCL specific cysteine proteinase. As shown in Fig.5, 5 μM castalagin considerably decreased the expression levels of RANK, c-fms, NFATc1, NFκB, c-Src, and cathepsin K in OCLs. However, 50 μM castalagin had weak inhibitory effects on the protein levels of c-fos in OCLs (Fig. 5A). Since the phosphorylated IκBα enhances its degradation and nuclear transport of NF-κB as a transcription factor, we next determined the effects of castalagin on NF-κB activation as nuclear translocation of NF-κB p65. Immunofluorescence microscopy analysis revealed that nuclear localization of NF-κB p65 was observed in RANKL-stimulated control (0 μM) BMMs (Fig. 5B). However, treatment with 25 or 50μM castalagin prevented the nuclear accumulation of NF-κB p65 in the cells (Fig. 5B). These results indicate that castalagin caused significantly down-regulation of many essential factors involved in OCL differentiation, including RANK, c-fms, NFκB, c-Src, NFATc1, and cathepsin K. Taken together with the data on signaling pathways, castalagin inhibited 6 major signaling pathways, including NFATc1, NF-κB, PI3K/Akt,

JNK, Erk, and p38 MAPK, and it hampered the expression of several OCL marker proteins.

Castalagin inhibits HO-1 expression in OCLs

Recently, we demonstrated that RANKL-induced suppression of HO-1 is required for osteoclastogenesis (Sakai *et al.*, 2011). Finally, to determine the molecular mechanisms by which castalagin inhibits OCL differentiation, we analyzed the effects of castalagin on the expression levels of HO-1. Conversely, induction of HO-1 by pharmacological compounds suppresses the OCL differentiation. As shown in Fig.6, the time course of mRNA levels of HO-1 in OCLs treated with 10 μM castalagin increased after a 3-h incubation period, but gradually decreased. Taken together, these results indicate that HO-1 induction by castalagin also participates in its inhibition of OCL differentiation.

Discussion

In this study, we demonstrated that castalagin inhibits the OCL differentiation from BMMs into mature OCLs, and markedly prohibited the bone resorbing activity of OCLs. Importantly, cell viability was maintained even at higher castalagin concentrations of 25 and 50 μM. Castalagin interfered with the Akt, Erk, JNK and p38 MAPK-dependent pathways. Castalagin significantly caused down-regulation of many essential factors involved in OCL differentiation, including RANK, c-fms, c-src, NFκB, NFATc1, and cathepsin K. As one of its molecular mechanisms, induction of HO-1 by castalagin also participated in the inhibition of osteoclastogenesis. Thus, this study is the first report, to our knowledge, that castalagin has an inhibitory effect on osteoclastogenesis.

The important pharmacological characteristic of castalagin is its inhibitory effects on osteoclastogenesis through a broad range of signaling pathways. Major signaling pathways for RANKL-induced OCL differentiation are known to include the NFATc1, NF-κB, PI3K/Akt, JNK, ERK, and p38 MAPK–dependent pathways (Darnay *et al*., 1999; Matsumoto *et al*., 2000; Zhang *et al*., 2001). Castalagin inhibits all the 6 of these major signaling pathways, although it strongly blocks phosphorylation of Akt, Erk, and p38 MAPK, and sufficiently reduces phosphorylation of JNK and IκBα. However, in

general, most natural compounds inhibiting osteoclastogenesis attenuates only some of these 6 major signaling pathways. For example, our previous studies indicated that kahweol, a coffee-specific diterpene, suppresses OCL differentiation through the abolishment of Erk phosphorylation, and partial inhibition of IκBα and Akt-dependent pathways, despite having no effect on the JNK and p38 MAPKs-dependent pathways (Fumimoto *et al.*, 2012). Fisetin, a natural flavonoid, inhibits osteoclastogenesis via intense inhibition of the Erk, Akt and JNK-dependent pathways, and partial inhibition of the IκBα, but not the p38 MAPKs-dependent pathway (Sakai *et al.*, 2013). Thus, inhibition by castalagin of all 6 major signaling pathways involved in OCL differentiation may lead to powerful inhibitory effects on osteoclastogenesis.

Despite its the multiple inhibitory effects on RANKL-induced OCL differentiation signaling, castalagin has low cytotoxicity. Indeed, castalagin had more than a 50 % inhibitory effect on osteoclastogenesis of BMMs at 1 μM, whereas it displayed no cytotoxicity, even at 50 μM (Fig. 2). For example, in our previous studies with similar analyses, kahweol has approximately 60 % inhibitory effects on osteoclastogenesis of BMMs at 10 μM, whereas it displays slightly cytotoxicity at 25 μM (Fumimoto *et al.*,

2012). Similarly, fisetin also displays approximately 80 % inhibitory effects on osteoclastogenesis of BMMs at 10 μM, but with significant cytotoxicity at 20 μM (Sakai *et al*., 2013). Thus, castalagin has a lower cytotoxicity compared with other natural compounds like kahweol and fisetin, although it has a powerful inhibitory effect on the osteoclastogenesis of BMMs.

To date, only a few of the ellagitannins have been shown to have inhibitory effects on OCL differentiation. Furosin, a hydrolyzable tannin, suppress RANKL-induced OCL differentiation from BMMs or macrophage cell line RAW264.7 cells via inhibition of JNK and p38-MAPKs (Park *et al.*, 2004). Recently, geraniin, a well-studied tannin, has been shown to reduce the number of mature OCLs and inhibit bone resorption activity, through inhibition of MMP-9 expression (He *et al*., 2013). However, those studies on the effects of furosin and geranin on osteoclastogenesis failed to report cell viabilities (Park *et al.*, 2004) (He *et al*., 2013). Therefore, the cytotoxicity of furosin or geranin on OCLs remains unknown. In this study, the lower concentration of castalagin significantly inhibited osteoclastogenesis, but cell viability was maintained even at the higher concentration, suggesting that it has powerful inhibitory effects on

osteoclastogenesis, but low cytotoxicity. At present, the most common therapeutic agents for bone metabolism are bisphosphonates, which have strong inhibitory effects on osteoclasts but with high toxicity. Therefore, the development of agents with lower toxicity is required. Castalagin and its analogs may provide a new ideal agent for osteoclast inhibition. This is because high doses or prolonged use of bisphosphonates, which are the most commonly used for osteoporosis, causes several side effects including osteonecrosis of the jaw, atypical femoral fracture, and musculoskeletal pain.

In conclusion, castalagin inhibits OCL differentiation through blocking a broad range of signaling pathways, but it has a lower cytotoxicity compared to other agents. The present study indicates that castalagin may have therapeutic effects in the treatment of bone diseases such as osteoporosis, periodontitis and rheumatoid arthritis.

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Figure Legends

Fig. 1. Structure of castalagin.

Fig. 2. Effects of castalagin on osteoclast (OCL) differentiation from bone marrow-derived macrophages (BMMs). A) BMMs were cultured for 72 h with 30

ng/mL macrophage colony-stimulating factor (M-CSF), 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL), and castalagin at the indicated concentrations. Tartrate-resistant acid phosphatase (TRAP) staining was performed. B) The number of TRAP-positive OCLs was counted. C) Cell viability of the BMM-derived-OCLs was analyzed using a Cell Counting Kit. Data are shown as mean ± standard deviation (significance compared with M-CSF and RANKL $^{*}P < 0.05$, $^{**}P < 0.01$). Results are representative of 3 independent experiments.

Fig. 3. Effects of castalagin on the bone resorbing activity of osteoclasts (OCLs). Bone marrow-derived macrophages (BMMs) were cultured with 30 ng/mL macrophage colony-stimulating factor (M-CSF), 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL), and castalagin at the indicated concentrations for 5 days. A**)** Photograph of the bone resorbing activity of OCLs. B) The resorption area was determined using ImageJ software (significance compared with the control. $^{**}P < 0.01$).

Fig. 4. Effects of castalagin on the essential signaling involved in osteoclast (OCL)

differentiation. A) Bone marrow-derived macrophages (BMMs) were cultured with 30 ng/mL macrophage colony-stimulating factor (M-CSF) for 12 h in the presence of a vehicle or 10 μM castalagin. The cells were subsequently stimulated with 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) for the indicated times (0, 5, 10, 15, and 30 min). The cell lysates were subjected to SDS-PAGE, followed by western blotting with antibodies to p-extracellular signal-regulated kinase (Erk), p-protein kinase B (Akt), p-p38, p-Jun N-terminal kinase (JNK), p-inhibitor of nuclear factor kappa B alpha (IκBα), and ß-actin. Results are representative of 3 independent experiments.

Fig. 5. Effects of castalagin on protein expression of osteoclast (OCL) marker proteins. A) Bone marrow-derived macrophages (BMMs) were cultured with 30 ng/mL macrophage colony-stimulating factor (M-CSF) and 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) for 72 h in the presence of castalagin at the indicated concentrations (0, 1, 5, 25, and 50 μM). The cell lysates were subjected to SDS-PAGE, followed by western blotting with antibodies specific to receptor activator

of nuclear factor kappa-B (RANK), c-fms, nuclear factor of activated T cells cytoplasmic-1 (NFATc1), nuclear factor kappa B (NF-κB), c-Fos, c-Src, cathepsin K, and ß-actin. B) BMMs were cultured with M-CSF (30 ng/mL) and RANKL(50ng/mL) for 72 h in the presence of 0-50 μM castalagin. For NF-κB localization, anti-p65 antibody was used as primary antibody, and followed by Alexa 488 conjugated-second antibody. Microscope images of fluorescence were digitized using LSM710 confocal laser microscope. Bar: 10μm

Fig. 6. Effects of castalagin on mRNA expression of heme oxygenase-1 (HO-1). Bone marrow-derived macrophages (BMMs) were cultured with 30 ng/mL macrophage colony-stimulating factor (M-CSF) and 50 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) for the indicated times in the absence or presence of 10 μM castalagin. The mRNA expression levels were determined by real time PCR using specific primers for *HO-1*. β-actin was used as a control. The graph shows the fold induction of each gene as compared to non-treated cells at 0 time point. Data are shown as mean ± standard deviation, and analyzed by 2-factor ANOVA (“a” indicates significant difference from corresponding 0 time point. “b” indicates significant

difference between non-treatment and castalagin treatment at the same time point.). Results are representative of 3 independent experiments.

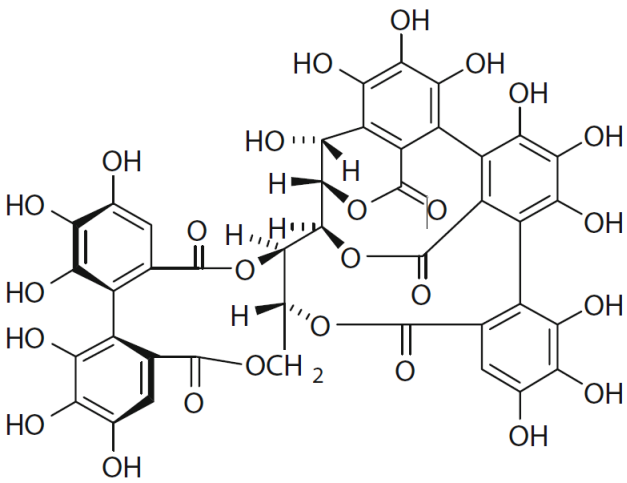
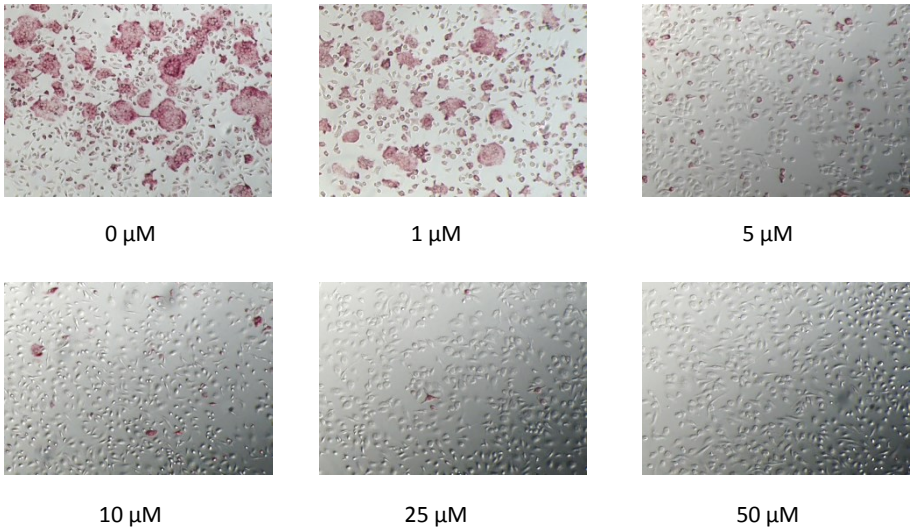
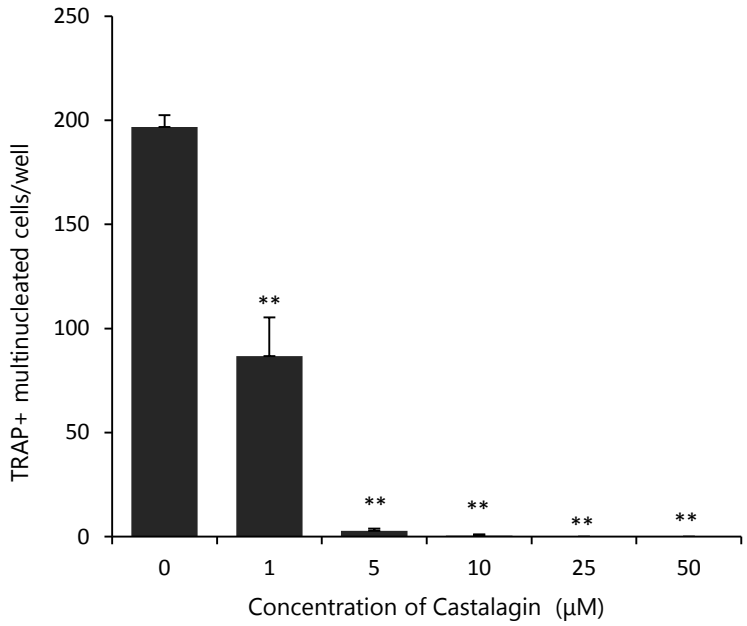


Fig. 1

A


0 μM
1 μM
5 μM
10 μM
25 μM
50 μM

B


TRAP+ multinucleated cells/well
250
200
150
100
50
0
**
**
**
**
0
1
5
10
25
50
Concentration of Castalagin (μM)

C

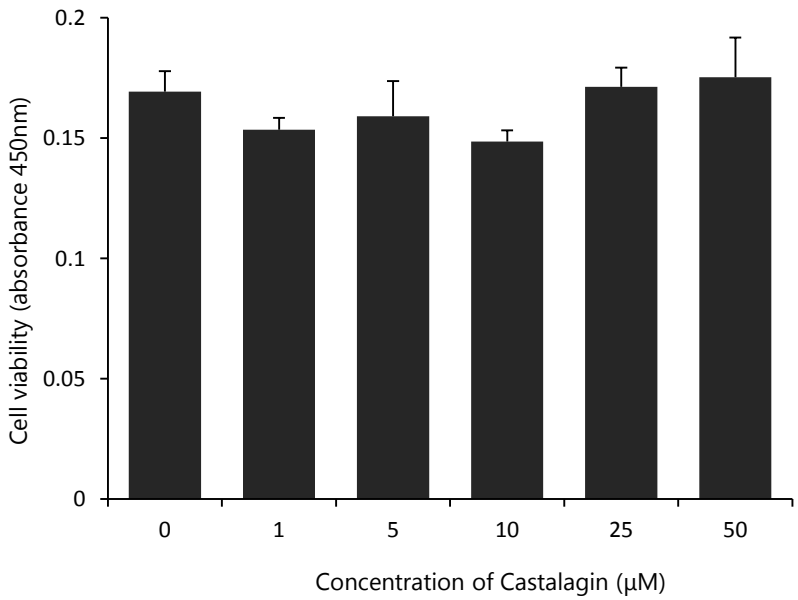
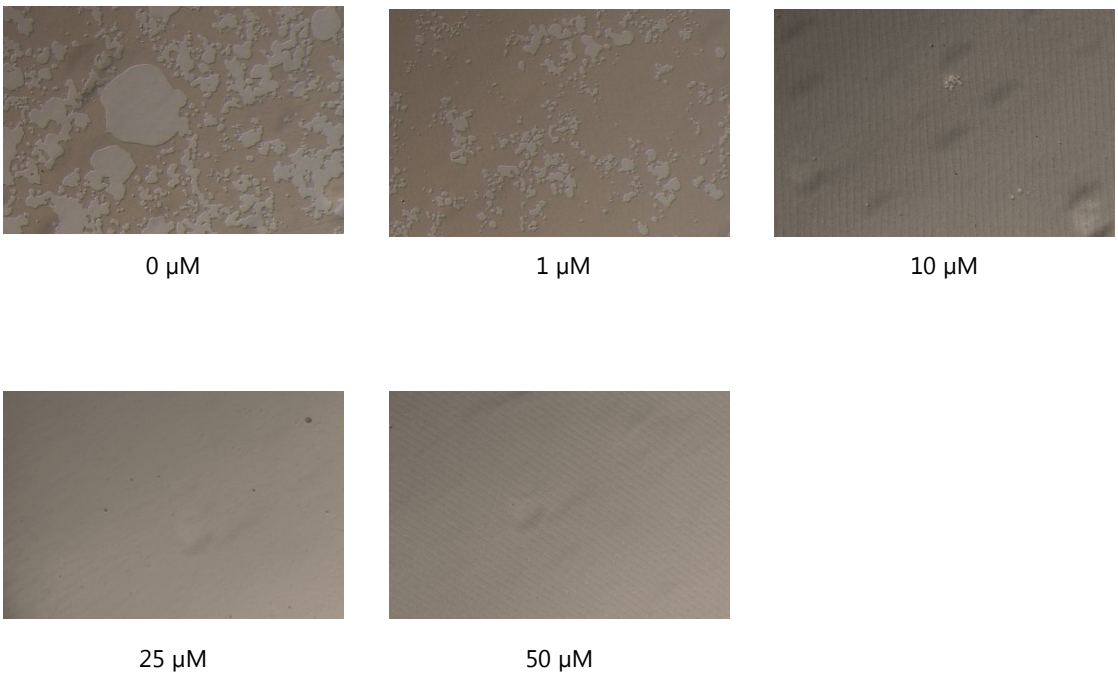

Cell viability (absorbance 450nm)
0.2
0.15
0.1
0.05
0
0
1
5
10
25
50
Concentration of Castalagin (μM)

Fig. 2

A


0 μM
1 μM
10 μM
25 μM
50 μM

B

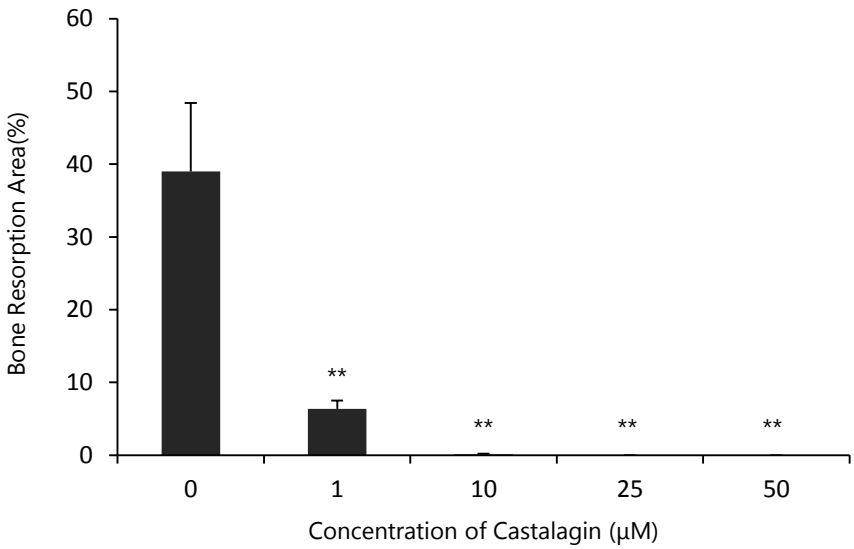

Bone Resorption Area(%)
60
50
40
30
20
10
0
**
**
**
**
0
1
10
25
50
Concentration of Castalagin (μM)

Fig. 3

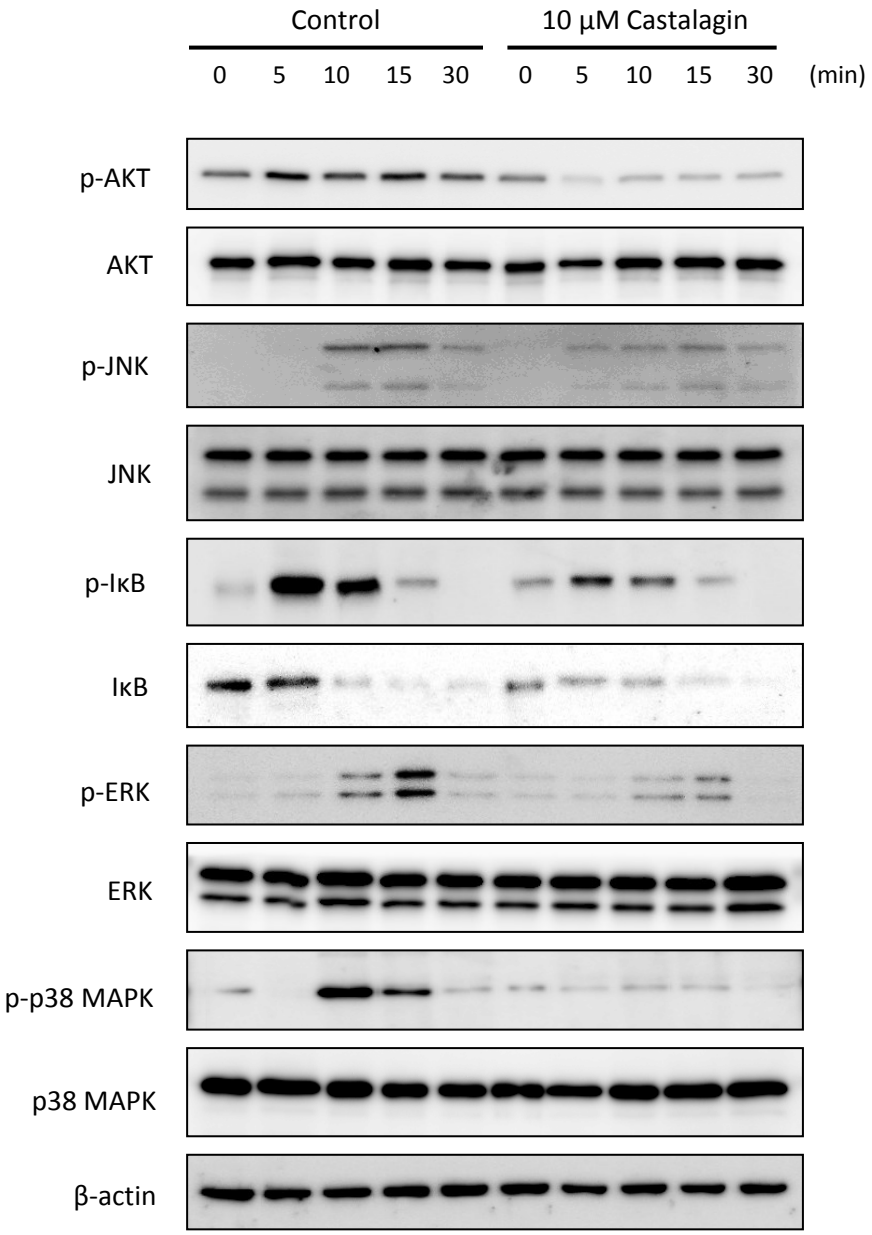
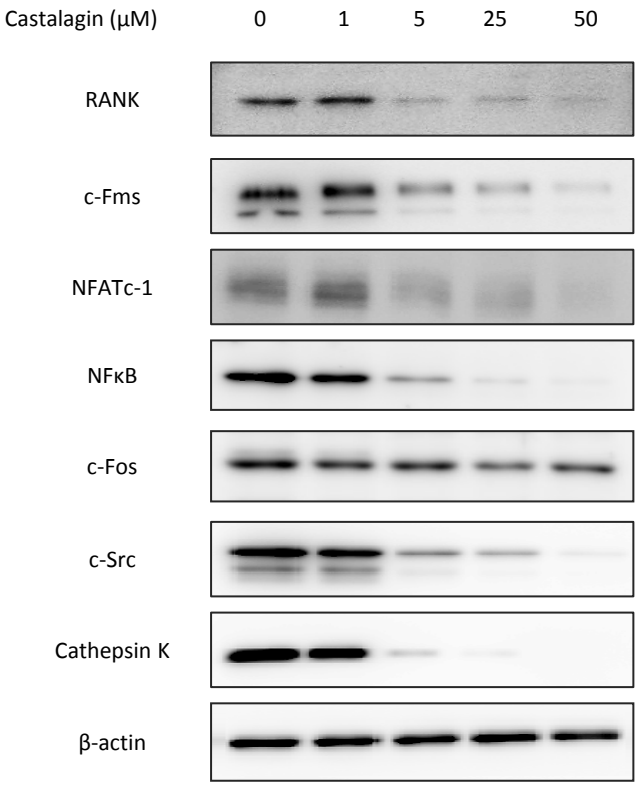

Control
10 μM Castalagin
0 5 10 15 30 0 5 10 15 30 (min)
p-AKT
AKT
p-JNK
JNK
p-IκB
IκB
p-ERK
ERK
p-p38 MAPK
p38 MAPK
β-actin

Fig. 4

A


Castalagin (μM)
0
1
5
25
50
RANK
c-Fms
NFATc-1
NFκB
c-Fos
c-Src
Cathepsin K
β-actin

B

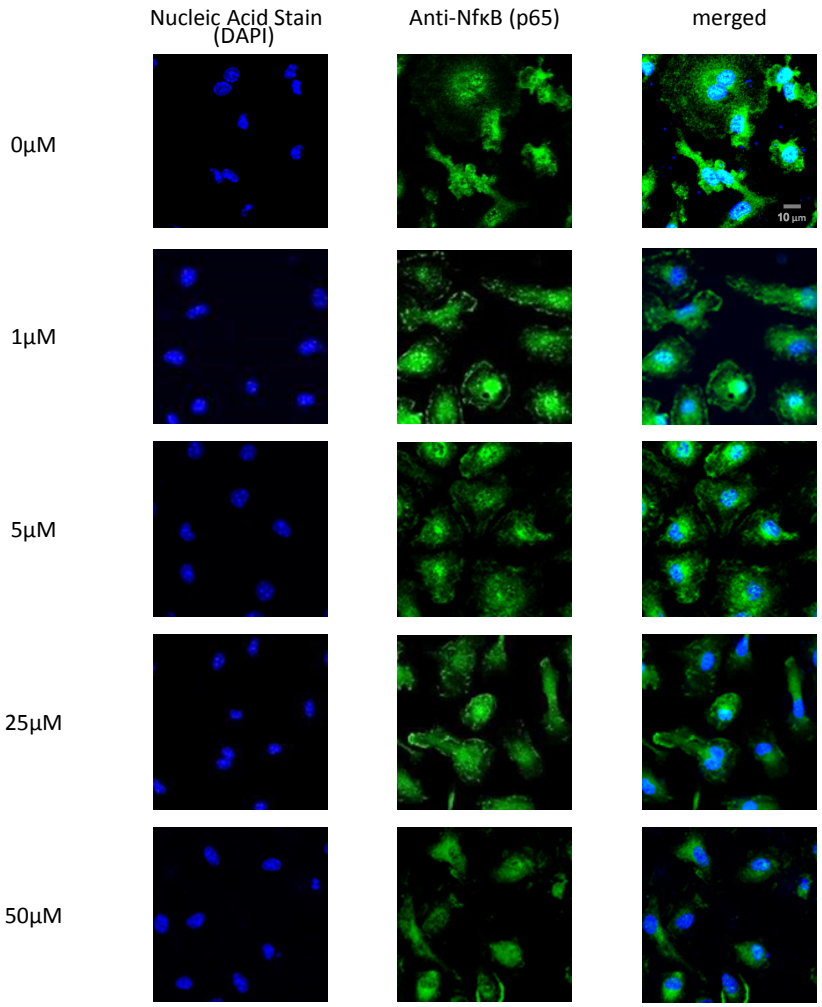

Nucleic Acid Stain (DAPI)
Anti-NfκB (p65)
merged
0μM
1μM
5μM
25μM
50μM
10 μm

Fig. 5

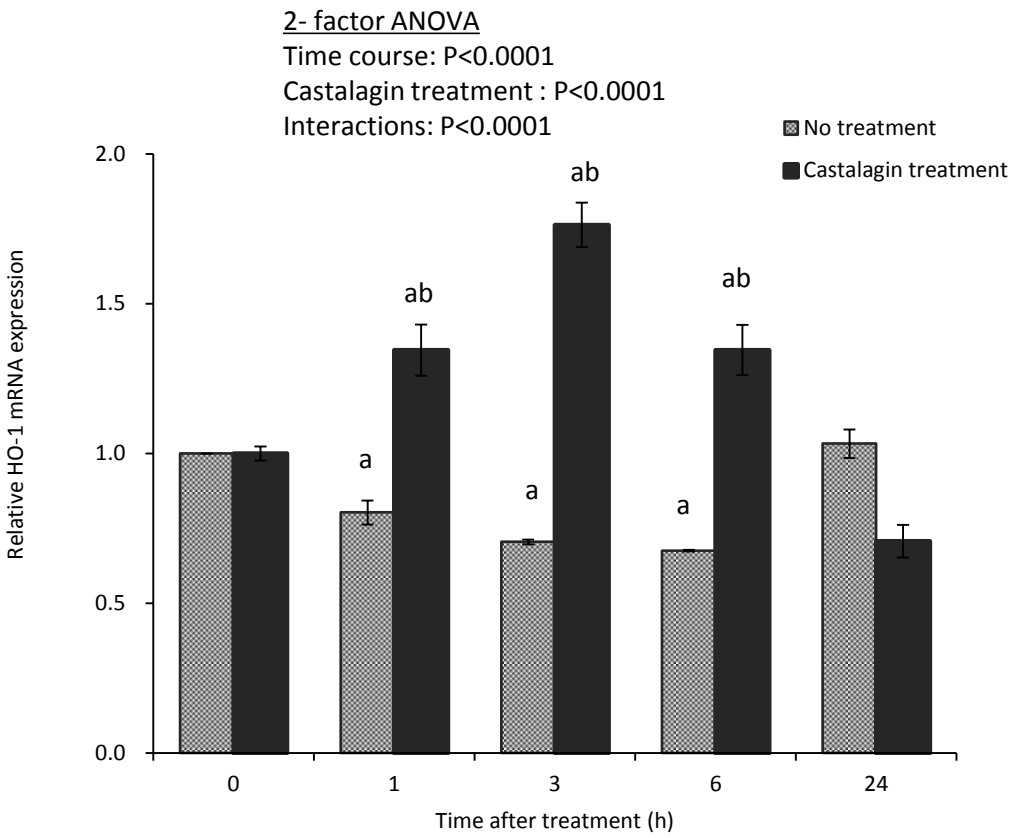

2- factor ANOVA
Time course: P<0.0001
Castalagin treatment : P<0.0001
Interactions: P<0.0001
No treatment
Castalagin treatment
Relative HO-1 mRNA expression
2.0
1.5
1.0
0.5
0.0
a
ab
a
ab
a
ab
0
1
3
6
24
Time after treatment (h)

Fig. 6