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Author(s)	大森, 晶奈
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博 士 論 文

Rosmarinic acid and arbutin suppress osteoclast differentiation by inhibiting superoxide and NFATc1 downregulation in RAW264.7 cells

（ロスマリン酸とアルブチンはスーパーオキシド除去作用と NFATc1 の発現抑制によって RAW264.7 細胞からの破骨細胞分化を抑制する）

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北海道大学

大学院歯学研究科口腔病態学専攻

大　森　晶　奈

ABSTRACT : We investigated the effect of the natural polyphenols rosmarinic acid and arbutin on osteoclast differentiation in RAW 264.7 cells. Rosmarinic acid and arbutin suppressed osteoclast differentiation and had no cytotoxic effect on osteoclast precursor cells. Rosmarinic acid and arbutin inhibited superoxide production and intracellular superoxide in a dose dependent manner. mRNA expression of the master regulator of osteoclastogenesis, nuclear factor of activated T cells cytoplasmic1 (NFATc1), and the osteoclast marker genes, matrix metalloproteinase-9, tartrate-resistant acid phosphatase and cathepsin-K, decreased after treatments with rosmarinic acid and arbutin. Furthermore, resorption activity decreased with the number of osteoclasts. These results suggest that rosmarinic acid and arbutin may be useful for the prevention and treatment of bone diseases such as osteoporosis through mechanisms involving inhibition of superoxide and down-regulation of NFATc1.

Key Words : rosmarinic acid, arbutin, osteoclast, superoxide, osteoclastogenesis

Introduction

Polyphenols are aromatic and slightly bitter-tasting compounds in vegetables, fruits and herbs. It has been reported that polyphenols can prevent several chronic diseases such as arteriosclerosis, cardiovascular diseases and cancer due to their antioxidant activity [1-3]. The most well-known study associated with polyphenols is the investigation called the French paradox. The French paradox is the epidemiological observation that French people have a low incidence of coronary heart disease despite a diet high in saturated fat, and is generally attributed to the high concentration of polyphenols in red wine [4,5].

Recent studies have shown that polyphenols not only prevent arteriosclerosis and cancer, but also affect bone diseases by suppressing osteoclast differentiation and increasing bone mineral density in ovariectomized mice [6,7]. Several polyphenols such as curcumin, resveratrol and plum polyphenol can scavenge reactive oxygen species (ROS) associated with osteoclast differentiation [8-10]. Rosmarinic acid is a natural polyphenol contained in Lamiaceae herbs such as *Perilla frutescens*, lemon balm mint, sage and sweet basil [11]. In particular, rosmarinic acid is effective against allergies, exhibits anti-inflammatory properties, and is used in health foods [12, 13]. Arbutin is a natural polyphenol contained in plants such as Ericaceae, Asteraceae and Vaccinium [14]. Arbutin has recently been reported to exert a potent inhibitory effect on the hydroxylation reaction of tyrosinase and has been widely used for skin whitening [15, 16].

Osteoclasts are members of the monocyte/macrophage lineage and are formed by fusion of their precursor cells. The receptor activator of nuclear factor-kB ligand (RANKL) is the most essential cytokine for the process of osteoclast differentiation and activation [17]. RANKL is a member of the tumor necrosis factor (TNF) family and is expressed as a membrane-bound protein in osteoblast and stromal cells. Binding of RANKL with the receptor RANK induces activation of TNF receptor-associated factor (TRAF6) and the c-Fos pathway. This activates downstream signaling pathways involving the nuclear factor of activated T cells cytoplasmic 1 (NFATc1) and leads to the formation of tartrate-resistant acid phosphatase (TRAP) positive osteoclasts [18].

The murine macrophage/osteoclast precursor cell line, RAW 264.7 (RAW), is a widely used pre-osteoclast model. RAW cells differentiate into osteoclasts in the presence of

RANKL. Bone is continuously and precisely remodeled by the coordination of bone-forming osteoblasts and bone-resorbing osteoclasts [19]. Osteoporosis is characterized by reduced bone mass and diminished bone integrity caused by an imbalance between osteoblasts and osteoclasts [20].

ROS contribute to the aging process and the etiology of various degenerative diseases, including osteoporosis [21]. Moreover, osteoclasts have been shown to be activated by ROS, resulting in enhanced bone resorption. On the other hand, ROS also play an important role as secondary messengers in osteoclast signaling pathways [22]. ROS are generated by superoxide production of NADPH oxidase in a process called the respiratory burst. Approximately 3-10% of the oxygen utilized by tissues is converted to ROS, including superoxides [23]. A recent study reported that rosmarinic acid inhibited phorbol myristate acetate (PMA)-induced superoxide production in a macrophage cell line [24]. Arbutin has been shown to inhibit ultraviolet A (UVA) irradiation-induced ROS in skin cells [25].

Rosmarinic acid has been shown to inhibit NF-κB activation during osteoclast formation [26]. However, with the exception of this mechanism of action of rosmarinic acid, the inhibitory effects of polyphenols on osteoclasts remain unknown. In particular, the therapeutic effects of arbutin on bone have not been studied. The purpose of this study was to investigate the effects of rosmarinic acid and arbutin on the differentiation and formation of osteoclasts from RAW cells through suppression of the superoxide-mediated signaling pathway. We hypothesized that these polyphenols would downregulate NFATc1, resulting in the direct inhibition of osteoclastogenesis, and lead to a decrease in osteoclast resorption activity.

Materials and methods

Chemicals. RAW cells were purchased from ATCC (Manassas, VA, USA). Rosmarinic acid, arbutin, Dulbecco's Modified Eagle's medium (DMEM), α-minimum essential medium (α-MEM), ethanol, Green Chemiluminescence CD, methanol, trifluoroacetic acid and sodium hypochlorite were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) and TRIzol were from Invitrogen (Carlsbad, CA). Ascorbic acid 2-phosphate, L-alanyl-L-glutamine and Fast Red Violet LB salt were obtained from Sigma-Aldrich (St. Louis, MO). RANKL was from Oriental Yeast (Tokyo, Japan).

Preparation of rosmarinic acid and arbutin. The structures of rosmarinic acid and arbutin are shown in Fig. 1. Rosmarinic acid was dissolved in ethanol. Arbutin was dissolved in deionized water. The reagents were sterilized by filtering through a 0.22 μm membrane filter. Stock solutions of rosmarinic acid and arbutin were stored at 4°C until use.

Cell culture. The murine monocyte/macrophage cell line RAW was used as osteoclast precursor cells. RAW cells differentiate into osteoclasts in the presence of RANKL (50 ng/ml). RAW cells were grown in DMEM medium supplemented with 10% heat-inactivated FBS and 66.7 μg/ml kanamycin-sulfate (Meiji Seika, Tokyo, Japan). The cells were seeded onto 100-mm standard dishes (BD Falcon, Franklin Lakes, NJ). After overnight culture, the cells were cultured in α-MEM supplemented with 10% heat-inactivated FBS, RANKL, 284 μM ascorbic acid 2-phosphate, 2 mM L-alanyl-L-glutamine and 66.7 μg/ml kanamycin-sulfate. They were then treated with or without rosmarinic acid or arbutin at the concentrations indicated below. These cultures were fed every other day by replacing the old medium with an identical volume of fresh medium. All cultures were incubated at 37°C in a humidified 5% CO_2, 95% air atmosphere.

MTT assay for cell viability. A Vialight^{TM} Kit (Lonza, Allendale, NJ) was used to measure the viability of RAW cells exposed to rosmarinic acid or arbutin. This kit utilizes the ATP present in live cells in a luciferase-catalyzed reaction to produce light. RAW cells were seeded on a 96-well plate (BD) at a density of 0.25×10^4 cells/well and treated with rosmarinic acid or arbutin without RANKL for 72 h. After culture, the cells were lysed in 50 μl of cell lysis solution. After incubation for 15 min at room temperature, the cell lysates were transferred to a luminescence plate (Nunc, Roskilde, Denmark) and mixed with 100 μl AMR PLUS reagent. After 2 min incubation at room temperature in the dark, the plate was read on a luminescence plate reader (Wallac 1420 ARVOsx, Perkin Elmer, Waltham, MA). The luminescence measured from each treatment group was converted to a percentage of the luminescence measured from the control cells (no rosmarinic acid or arbutin added).

Tartrate-resistant acid phosphatase (TRAP) staining. Osteoclasts were assessed by cytochemical staining for TRAP. RAW cells were seeded on a 48-well plate (BD) at a density of 0.5×10^4 cells/well and treated with RANKL and rosmarinic acid or arbutin. After 6 days, the cells were fixed with 10% neutral formalin. They were then washed with distilled water and stained in TRAP staining solution (pH 5.0) with Fast Red Violet LB salt [27]. TRAP-positive osteoclasts with more than 2 nuclei were considered to be osteoclasts. Osteoclasts with 8 nuclei or more were considered to be large osteoclasts. The number of osteoclasts was counted under a light microscope.

Measurement of superoxide. Superoxide concentration was measured using a Green Chemiluminescence CD kit according to the manufacturer's instructions. Green Chemiluminescent CD is a highly sensitive chemiluminescence probe which reacts with the superoxide anion and a luminescence dye specific for the detection of superoxide. Briefly, 0.5×10^4 cells/well were seeded on a 48-well plate and treated with RANKL and rosmarinic acid or arbutin for 48 h. The contents of the kit were dissolved in hot methanol:water 1:1 containing 0.1% (w/v) trifluoroacetic acid. To measure the superoxide produced in the medium, 200 μl of the medium was mixed with 150 μl of the reagent. Cells were gently washed twice with PBS. Thereafter, the intracellular superoxide was measured by mixing 150 μl of the reagent and 200 μl of the cell suspension. The luminescence intensity of the samples was measured using a

luminescence plate reader. The value for each treated group was converted to a percentage of the control luminescence.

Superoxide dismutase (SOD) activity. The SOD activity of the culture medium was measured using a SOD Assay Kit-WST according to the manufacturer's protocol (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, the RAW cells were seeded on a 96-well plate at a density of 0.25×10^4 cells/well and treated with RANKL and rosmarinic acid or arbutin for 48 h. The SOD activity was measured by mixing the reagents from 220μl of the WST kit with 20μl of the culture medium. After incubation for 20 min at 37°C, absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 680, Hercules, CA, USA). The value for each treated group was converted to a percentage of the value obtained for the control group.

Real-Time Quantitative PCR. RAW cells were seeded on a 6-well plate at a density of 6×10^4 cells/well and treated with RANKL and rosmarinic acid or arbutin for 24 h. After incubation, total RNA was isolated using TRIzol according to the manufacturer's instructions. cDNA synthesis was performed in 25 μl from 1 μg of total RNA using ReverTra Ace reverse transcriptase (Toyobo Co., Osaka, Japan) and oligo dT primers (Toyobo).

The specific primer sets used for NFATc1 (Mm00479445_m1), matrix metalloproteinase-9 (MMP-9) (Mm00442991_m1), TRAP (Mm00475698_m1), cathepsin-K (Mm00484036_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1) were designed using the Primer Express program (Applied Biosystems, Foster City, CA, USA) and were purchased from Applied Biosystems. Quantitative real-time PCR was performed using an ABI7300 (Applied Biosystems). We used the comparative Ct method to calculate mRNA expression and verified the Ct values of both the calibrator and the samples of interest by normalizing to GAPDH. The comparative Ct method is also known as the $2^{-\Delta\Delta Ct}$ method, where, $\Delta Ct = \Delta Ct, \text{sample} - \Delta Ct, \text{reference}$. $\Delta CT, \text{sample}$, is the Ct value for the sample normalized to the endogenous housekeeping gene, and $\Delta Ct, \text{reference}$, is the Ct value for the calibrator, which was also normalized to the endogenous housekeeping gene.

Resorption pit assay. A pit formation assay was conducted using a Corning Osteoassay Surface plate (Corning, Inc., Corning, NY, USA). RAW cells were seeded on plates at a density of 1×10^4 cells/well and treated with RANKL and rosmarinic acid or arbutin for 11 days. Cells were removed with 6% sodium hypochlorite solution. After washing the wells with pure water and allowing them to dry, the wells were observed under a light microscope and the pit area was compared with control.

Statistical analysis. All data are presented as mean ± standard deviation. Data were analyzed with the Dunnet test using Stat-View (version 5.0, SAS Institute Inc., Cary, NC, USA). Results with $p < 0.05$ and < 0.01 were considered statistically significant.

Results

Effect of rosmarinic acid and arbutin on cell viability in osteoclast precursor cells. In order to investigate the potential cytotoxicity of rosmarinic acid and arbutin on RAW cells as osteoclast precursors, cell viability was examined using the MTT assay (Fig. 2). Raw cells were incubated with different concentrations of rosmarinic acid or arbutin for 72 h. Rosmarinic acid at 20, 40, 60 and 100 μM (Fig. 2A) and arbutin at 50, 75, 100 and 200 μM (Fig. 2B) did not affect growth, as compared with the control cells. The viability of the cells at all the concentrations tested was >95%.

Rosmarinic acid and arbutin inhibited RANKL-induced osteoclast differentiation. Osteoclast differentiation was dose-dependently inhibited by rosmarinic acid and arbutin (Figs. 3A and B), as shown in Figs. 3C and D. The presence of rosmarinic acid at 20, 40, 60 and 100 μM decreased the number of TRAP-positive cells to about 78, 37, 6 and 0% of the control level, respectively (Fig. 3A). The presence of arbutin at 25, 50, 75, 100 and 200 μM decreased the number of TRAP-positive osteoclasts to about 78, 48, 35, 11 and 0% of the control level, respectively (Fig. 3B). The number of TRAP-positive large osteoclasts decreased with increasing concentration of rosmarinic acid (Fig. 3C) and arbutin (Fig. 3D).

Rosmarinic acid and arbutin inhibited superoxide production and SOD activity. The effect of rosmarinic acid or arbutin on superoxide production (Figs. 4A and B) and intracellular superoxide (Figs. 4C and D) in osteoclasts was investigated. RAW cells were incubated with rosmarinic acid (20, 40 and 60 μM) or arbutin (50, 75, and 100 μM) for 48 h. Rosmarinic acid and arbutin decreased superoxide production and intracellular superoxide in a dose-dependent manner. On the other hand, all concentrations of rosmarinic acid and arbutin tested had no effect on SOD inhibition (Fig. 4E and F).

Effect of rosmarinic acid and arbutin on mRNA expression of osteoclastic specific genes. Rosmarinic acid and arbutin suppressed RANKL-induced expression of osteoclastogenesis in a dose-dependent manner after 24 h.

Rosmarinic acid and arbutin inhibited RANKL-induced resorption by the pit area. Rosmarinic acid and arbutin decreased resorption by the pit area in a dose-dependent manner (Fig. 6A and B). Resorption by the pit area decreased in line with the number of osteoclasts, suggesting that rosmarinic acid and arbutin primarily affect osteoclast differentiation as opposed to activity.

Discussion

We reported that rosmarinic acid and arbutin at non-toxic concentrations suppress the number of TRAP-positive osteoclasts and large osteoclasts from RAW cells. The results clearly suggest that rosmarinic acid and arbutin suppress osteoclast formation by directly acting on osteoclast precursor cells. Rosmarinic acid has previously been reported to suppress osteoclast differentiation of murine bone marrow-derived macrophages (BMM) and RAW cells [26]. However, the regulation by rosmarinic acid of osteoclast differentiation needs to be further defined. Arbutin has not previously been reported to inhibit osteoclast differentiation or to show a beneficial effect on bone. We report for the first time that arbutin inhibits osteoclast differentiation of RAW cells. Specially, the inhibitory effects of rosmarinic acid at 20 μM concentration is similar to the inhibitory effect of arbutin at 25 μM: 21.54% caused by 20 μM rosmarinic acid and 22.19% caused by 25 μM arbutin. Furthermore, rosmarinic acid has been reported to be more potent for ROS removal compared to arbutin in the mesenteric artery in rats [28]. Therefore, we investigated whether the antioxidant reaction of rosmarinic acid and arbutin can regulate osteoclast differentiation.

ROS was recently recognized as a second messenger in the differentiation of osteoclasts and plays an important role in differentiation [22]. On the other hand, excessive ROS production results in an abnormal osteoclast phenomenon: the activation of osteoclast signals due to an increase in the number of osteoclasts.[29] Moreover, ROS production by osteoporotic bone tissue has been shown to be significantly higher than in normal bone tissue [30]. Several studies have reported that the ROS level increases during RANKL-induced osteoclast differentiation, and that this ROS generation can be attenuated by polyphenols such as curcumin and resveratrol [8, 9]. In addition, the antioxidants scopoletin and scopolin inhibit ROS and superoxide production in a dose-dependent manner [31]. A superoxide is converted into various ROS such as hydroxyl radical ($^{\bullet}$OH), hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO-) in the process of formation of the osteoclasts [32, 33]. Thus, to investigate whether rosmarinic acid and arbutin can inhibit superoxide generation during osteoclast differentiation, the production of superoxide and the intracellular levels of superoxide were analyzed. According to the sucopoletin and scopolin study referred to above, the intracellular ROS level increased to its highest value within 48 h and then decreased [31]. Therefore, we also analyzed superoxide levels after treating the cells with rosmarinic acid and arbutin

for 48 h and showed that rosmarinic acid and arbutin reduced superoxide production and intracellular superoxide in a dose-dependent manner. Most importantly, rosmarinic acid and arbutin inhibited superoxide, but had no effect on RANKL-induced SOD activity, suggesting rosmarinic acid and arbutin have a positive effect on RANKL-induced superoxide removal. The antioxidant activity of rosmarinic acid and arbutin have been observed using various cells. Rosmarinic acid inhibits ROS production in several cell lines such as dopaminergic cells and hematoma cells [34,35]. Rosmarinic acid also inhibits PMA-induced intracellular superoxide in macrophage cell lines, and has no effect on Cu/Zn SOD [24]. Arbutin suppresses UVA-induced ROS on cellular tyrosinase activity, primarily in skin cells such as human and mice melanocytes [25,36]. Additionally, curcumin suppresses phosphor I-κBα and intracellular ROS production in the osteoclast differentiation process in a dose-dependent manner [8]. Curcumin also completely suppresses NF-κB activity in the osteoclast differentiation process [37]. Rosmarinic acid shares common structural features with curcumin, so we can speculate that rosmarinic acid acts by a similar mechanism to inhibit RANKL-induced production of ROS such as superoxide. Furthermore, it was previously demonstrated that rosmarinic acid inhibits RANKL-induced NF-κB activation in the osteoclast differentiation process in BMM [26]. Arbutin suppresses NF-κB activity in BV2 microglial cells [38]. Further investigation is required to ascertain whether inhibition of NF-κB activity plays a role in suppression of osteoclast differentiation from RAW cells by rosmarinic acid and arbutin.

Taken together, our results show that rosmarinic acid and arbutin decrease RANKL-induced expression of NFATc1 mRNA. NFATc1 is a master regulator of RANKL-induced osteoclast differentiation [39]. NFATc1 plays a pivotal role in osteoclast activation via up-regulation of various genes in a series processes, such as osteoclast adhesion, migration, acidification and degradation of inorganic and organic bone matrix [18]. Thus, we investigated the effect of rosmarinic acid and arbutin on the mRNA expression of the osteoclast marker genes, MMP-9, TRAP and cathepsin-K, in osteoclasts. The mRNA expression of MMP-9, TRAP and cathepsin-K also were suppressed by the addition of rosmarinic acid and arbutin. MMP-9 is essential for initiating the osteoclastic resorption process by removing the collagenous layer from the bone surface prior to demineralization [40]. Furthermore, expression of MMP-9 in osteoclast is markedly higher than in other cell types [41]. Tea polyphenols suppress

osteoclast formation and activity by inhibiting the production of free radicals and MMP-9, and inducing apoptosis [42]. TRAP is a marker for osteoclasts, and there is increasing evidence of its proteolytic role in bone resorption [43]. TRAP knock-out mice have shown that bone shape and modeling are altered by increased mineral density, suggesting that TRAP plays an important role in bone resorption [44]. Cathepsin-K activity is required for the initial formation of actin rings and thus for the activation of osteoclasts [45]. A NFATc1 knock-out mouse study has reported that the endocardia expression of NFATc1 is dependent on the expression of cathepsin-K [46]. Based on these findings, we hypothesized that rosmarinic acid and arbutin might suppress the resorption activity of osteoclasts. To investigate the effect of resorption activity of osteoclasts treated with rosmarinic acid and arbutin, we treated cells with rosmarinic acid and arbutin after 11 days of culture using Corning osteo assay surface plates. Pit assays compared the pit area after treatment with rosmarinic acid and arbutin to that of the controls, suggesting that rosmarinic acid and arbutin impair the resorption activity of osteoclasts.

It is critical to investigate the effect of compounds *in vivo* rather than *in vitro*. More studies are therefore required to test the beneficial bone effects of rosmarinic acid and arbutin in animal models and human studies. From the viewpoint of clinical applications, it has been reported that the concentration of rosmarinic acid in the plasma of goat kids fed a diet containing 140.44 mg/kg or 273.86 mg/kg rosmarinic acid for 224 days was 0.78 mg/L and 0.81 mg/L, respectively [47]. Our results suggest that such plasma concentrations can significantly inhibit osteoclasts. Rosmarinic acid, as well as other polyphenols such as caffeic acid and ferulic acid, is metabolized when taken orally [48]. A rat serum concentration of 1.36 μmol/min/L was obtained by dosing 100 μmol/kg/body weight rosmarinic acid [49]. These reports apparently show the effective concentration difference between *in vitro* and blood concentrations. The blood levels of arbutin have not been reported. However, arbutin is used as a traditional medicine for treating urinary tract infections, thereby confirming its safety when taken orally [50].

In summary, we analyzed the effect of rosmarinic acid and arbutin on osteoclast differentiation by studying their inhibition of RANKL-induced superoxide, which is the source of ROS generation. Rosmarinic acid and arbutin inhibited osteoclast formation by blocking osteoclast marker genes such as MMP-9, TRAP and chathepsin-K via downregulation of NFATc1. These findings strongly suggest that rosmarinic acid and

arbutin hold promise for the treatment of various bone diseases such as osteoporosis and bone metastasis associated with excessive bone resorption.

Abbreviations

RAW cell	murine macrophage/osteoclast precursor cell line RAW 264.7 cell
RANKL	receptor activator of nuclear factor-κB (RANK) ligand
ROS	reactive oxygen species
SOD	superoxide dismutase
NFATc1	nuclear factor of activated T cells cytoplasmic 1
TRAP	tartrate-resistant acid phosphatase
GAPDH	glyceraldehydes-3-phosphatedehydrogenase
NADPH	nicotinamide adenine dinucleotide phosphate
NF- κB	nuclear factor-kappa B
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum

Conflint of Interest

The authors declare no competing financial interest.

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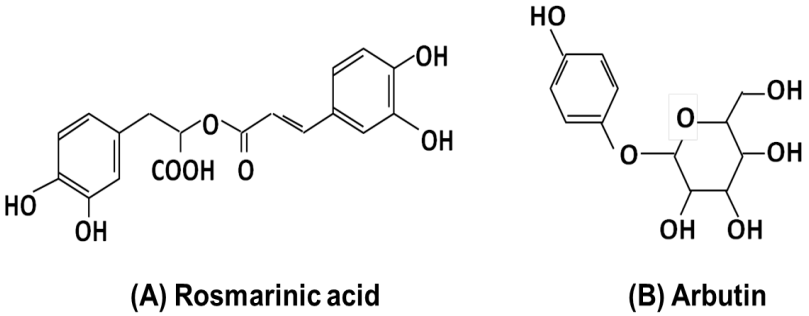
Figures.

Fig. 1.



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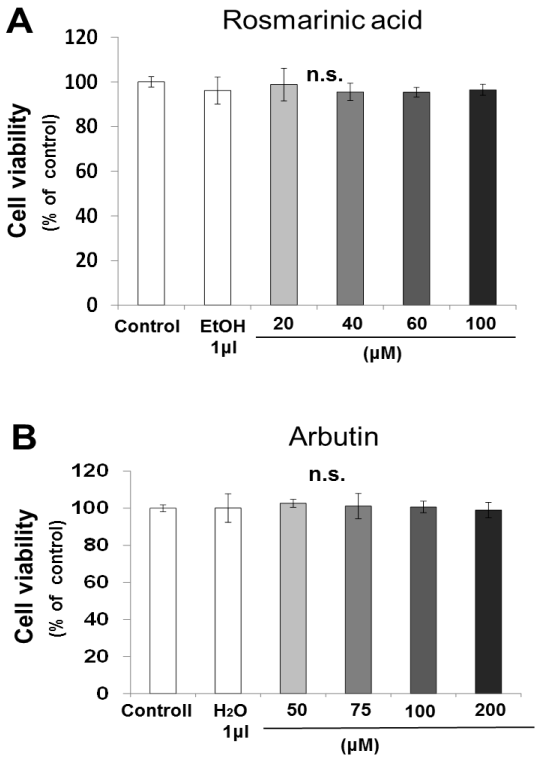

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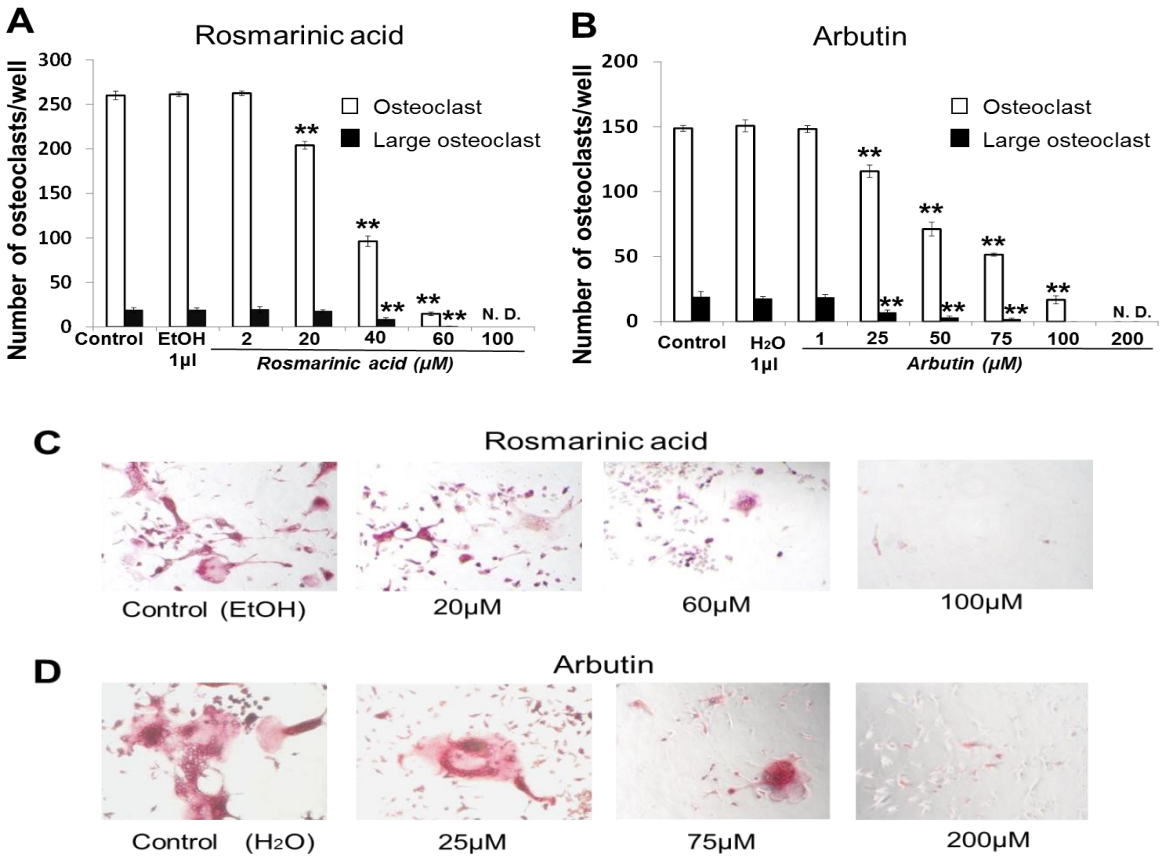
A
Rosmarinic acid
Number of osteoclasts/well
300
250
200
150
100
50
0
Osteoclast
Large osteoclast
**
**
**
**
N. D.
Control
EtOH 1μl
2
20
40
60
100
Rosmarinic acid (μM)
B
Arbutin
Number of osteoclasts/well
200
150
100
50
0
Osteoclast
Large osteoclast
**
**
**
**
**
N. D.
Control
H2O 1μl
1
25
50
75
100
200
Arbutin (μM)
C
Rosmarinic acid
Control (EtOH)
20μM
60μM
100μM
D
Arbutin
Control (H2O)
25μM
75μM
200μM

Fig. 4.

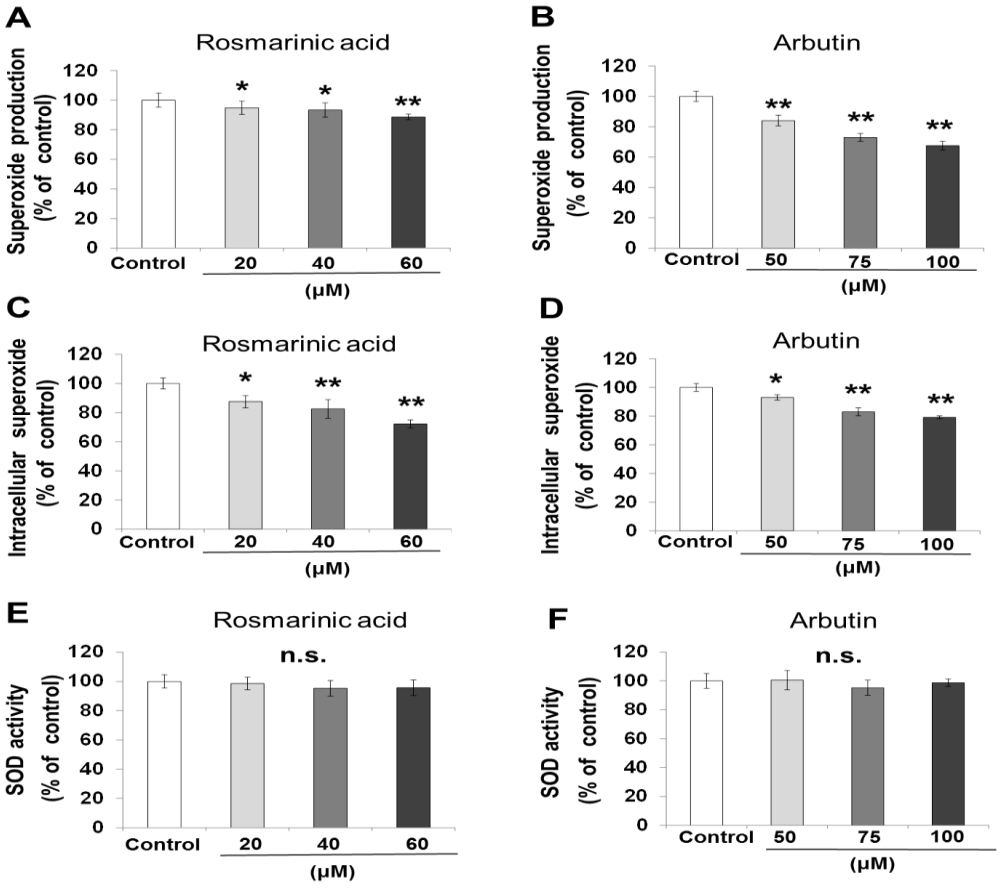
A
Rosmarinic acid
Superoxide production (% of control)
120
100
80
60
40
20
0
*
*
**
Control
20
40
60
(μM)
B
Arbutin
Superoxide production (% of control)
**
**
**
Control
50
75
100
(μM)
C
Rosmarinic acid
Intracellular superoxide (% of control)
*
**
**
Control
20
40
60
(μM)
D
Arbutin
Intracellular superoxide (% of control)
*
**
**
Control
50
75
100
(μM)
E
Rosmarinic acid
SOD activity (% of control)
n.s.
Control
20
40
60
(μM)
F
Arbutin
SOD activity (% of control)
n.s.
Control
50
75
100
(μM)

Fig. 5.

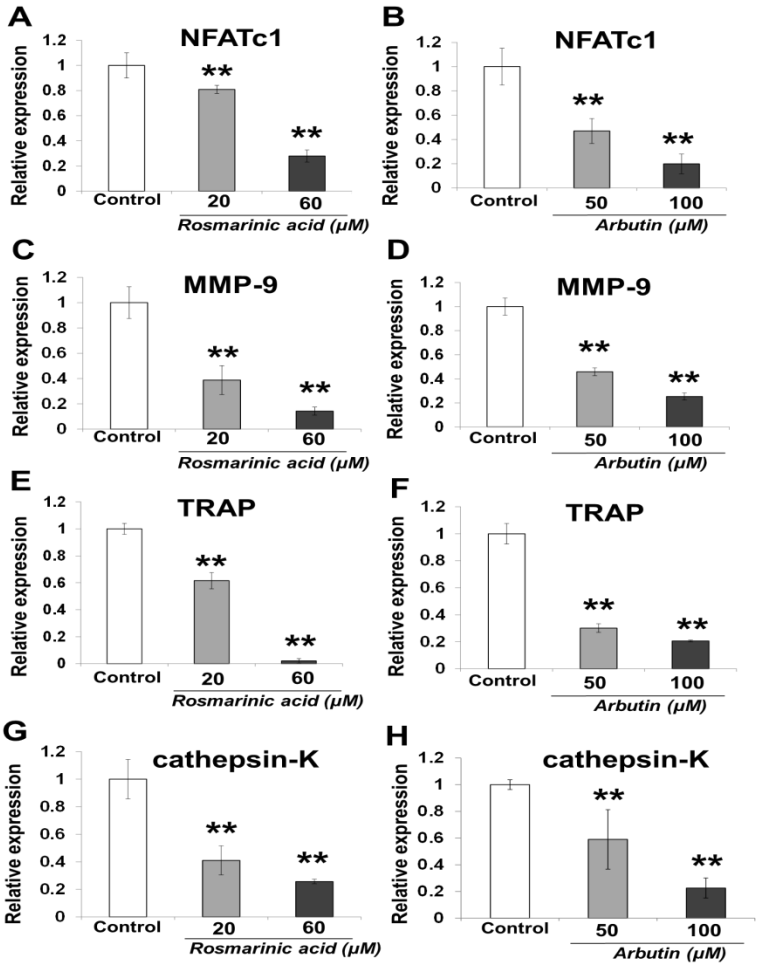
A
NFATc1
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Control
20
60
Rosmarinic acid (μM)
B
NFATc1
Relative expression
**
**
Control
50
100
Arbutin (μM)
C
MMP-9
Relative expression
**
**
Control
20
60
Rosmarinic acid (μM)
D
MMP-9
Relative expression
**
**
Control
50
100
Arbutin (μM)
E
TRAP
Relative expression
**
**
Control
20
60
Rosmarinic acid (μM)
F
TRAP
Relative expression
**
**
Control
50
100
Arbutin (μM)
G
cathepsin-K
Relative expression
**
**
Control
20
60
Rosmarinic acid (μM)
H
cathepsin-K
Relative expression
**
**
Control
50
100
Arbutin (μM)

Fig.6

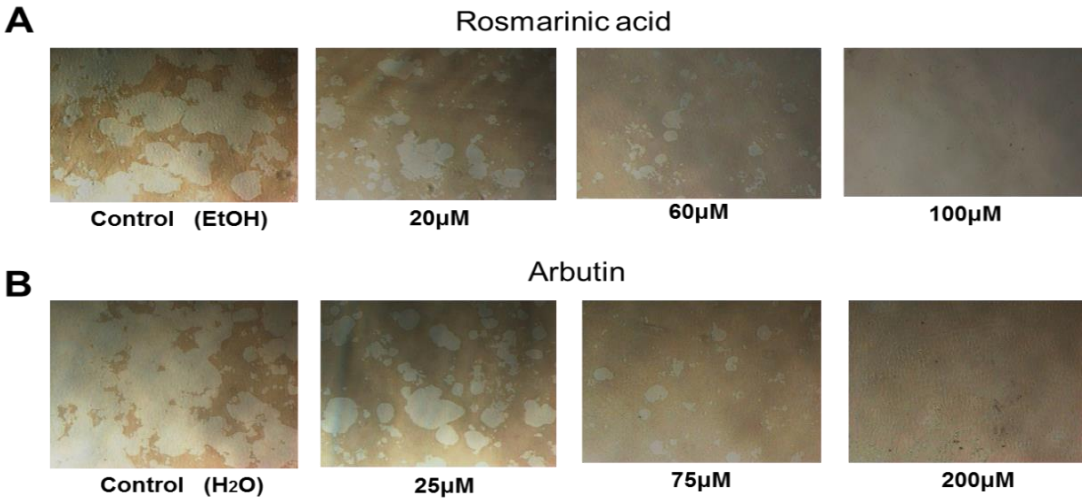
A
Rosmarinic acid
Control (EtOH)
20μM
60μM
100μM
B
Arbutin
Control (H2O)
25μM
75μM
200μM

Figure legends

Fig. 1. Structure of rosmarinic acid (A) and arbutin (B).

Fig. 2. Effect of rosmarinic acid and arbutin on RAW cell viability.

RAW cells were treated with RANKL and rosmarinic acid (A) or arbutin (B) at the indicated concentrations for 72 h, then the luminescence intensity was determined. Data are representative of results from three separate experiments. Data are expressed as percentages of the value of the control cells (means ± standard deviations, n = 5). (*$p < 0.05$, **$p < 0.01$, vs control).

Fig. 3. Inhibitory effect of rosmarinic acid and arbutin on RANKL-induced osteoclasts from RAW cells.

RAW cells were treated with RANKL and rosmarinic acid or arbutin at the indicated concentrations. After 6 days in culture, the cells were fixed and stained for TRAP. TRAP-positive multinucleated ($2 \leq$ nuclei) osteoclasts and TRAP-positive large multinucleated (8 < nuclei) osteoclasts were counted under a microscope (A and B). TRAP-positive osteoclasts were visualized by light microphotography (C and D). Data are representative of results from three separate experiments. Data are expressed as percentages of the value of the control cells (means ± standard deviations, n = 5). (*$p < 0.05$, **$p < 0.01$, vs control).

Fig. 4. Effect of rosmarinic acid and arbutin on superoxide production, intracellular superoxide and SOD activity.

RAW cells were cultured with RANKL and the indicated concentration of rosmarinic acid or arbutin for 48 h. Superoxide production (A and B) and intracellular superoxide (C and D) were measured by a luminescence intensity assay on using a luminescence plate reader. SOD activity (E and F) was measured at 450 nm using a microplate reader. Data are representative of results from four separate experiments. Data are expressed as percentages of the value of the control cells (means ± standard deviations, n = 5). (*$p < 0.05$, **$p < 0.01$, vs control).

Fig. 5. Expression of mRNAs for the master regulator of osteoclastogenesis, NFATc1, and the osteoclast-specific marker genes, MMP-9, TRAP and cathepsin-K by real time RT-PCR.

RAW cells were seeded on a 6-well plate at a density of 6×10^4 cells/well and treated with RANKL and rosmarinic acid or arbutin at the indicated concentration for 24 h. NFATc1 (A and B), MMP-9 (C and D), TRAP (E and F) and cathepsin-K (G and H) expression were analyzed by quantitative real-time RT-PCR as described in Materials and Methods. Results are shown as fold-change or relative quantitation of target expression ($2^{-\Delta\Delta Ct}$ method) relative to the control after normalization against GAPDH expression. Data are representative of results from four separate experiments. Data are expressed as percentages of the value of the control cells (means ± standard deviations, n = 5). (*$p < 0.05$, **$p < 0.01$, vs control).

Fig. 6. Inhibitory effect of rosmarinic acid and arbutin on RANKL-induced resorption pit area.

RAW cells were seeded on a 24-well plate at a density of 1×10^4 cells/well and treated with RANKL and rosmarinic acid or arbutin at the indicated concentration. After 11 days culture, the cells were washed with PBS and removed from the well walls with 6% sodium hypochlorite solution. The resorption pit area was photographed. Data are representative of results from three separate experiments. (n = 3).