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Inhibitory Effects of *Saururus Chinensis* Extracts on Osteoclast Differentiation

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Abstract – *Saururus chinensis* is a commonly used folk herb for the treatment of edema and liver diseases in Korea. To study the biological activity of *Saururus chinensis* in bone metabolism, we evaluated the effect of its extracts on osteoclast differentiation *in vitro* using primary mouse bone marrow-derived macrophages. Methanol extract (ME) from dried roots of *Saururus chinensis* was partitioned into methylene chloride (MF), ethyl acetate (EF), *n*-butanol (BF) and water fractions (WF). Tartrate-resistance acid phosphatase (TRAP) activity assay and western blot analysis were performed to determine the effect on osteoclast differentiation and mitogen-activated protein (MAP) kinases activation. ME, MF and EF dramatically inhibited receptor activator of NF-κB ligand (RANKL)-induced formation of multinucleated osteoclasts and activation of MAP kinases. This study firstly demonstrated that ME, MF and EF of *Saururus chinensis* have the potential to inhibit the osteoclast differentiation, which results from the inhibition of MAP kinases activations in part.
Keywords – *Saururus chinensis,* osteoclast differentiation, mitogen-activated protein kinases

Introduction

Osteoclasts are multinucleated cells responsible for the resorption of bone. The differentiation and activation of osteoclasts play an important role in the maintenance of bone mass, but excessive osteoclastic bone resorption can lead to several bone disorders such as osteoporosis. One of key molecule for osteoclasts differentiation is the receptor activator of NF-κB ligand (RANKL). The binding of RANKL to its receptor, RANK, leads to the recruitment of TNF receptor associated factor (TRAF) (Teitelbaum, 2000; Harada and Rodan, 2003) that subsequently trigger the signal transduction to the downstream molecules including mitogen-activated protein (MAP) kinases (Lee *et al.*, 2002; Lee and Kim, 2003). The activation of MAP kinases consequently regulates the expression of genes necessary for osteoclast differentiation by modulating the activation of transcription factors.

Natural products and dietary components containing herbal extracts have beneficial effects on the skeleton by influencing the processes of bone remodeling, particularly by inhibiting bone resorption (Putnam *et al.*, 2007). *Saururus chinensis* distributed in China and Korea is a traditionally used folk herb for the treatment of edema and liver diseases (Chung, 1998). To date, compounds isolated from *Saururus chinensis* and its extracts have been reported to have several biological activities including anti-oxidant (Lee *et al.*, 2004), anti-cancer (Song *et al.*, 2005) and immunosuppressive activity (Park *et al.*, 2007). Recently, sauchinone and saucerneol, lignans isolated from *n*-hexane or methylene chloride fraction of *Saururus chinensis*, have been reported to inhibit osteoclast differentiation and bone resorption (Han *et al.*, 2007, Kim *et al.*, in press). In a previous study, we also found that one more compounds isolated from *Saururus chinensis* inhibited osteoclastogenesis (unpublished data). These made us evaluate the effect of *Saururus chinensis* extracts on osteoclast differentiation in this study.

Experimental

Plant materials – The dried *Saururus chinensis* was purchased from Kyoung Dong Company (Seoul, Republic of Korea) and identified by a botanist, Prof. K. Bae (Chungnam National University, Daejeon, Republic of Korea).

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Extraction of *Saururus chinensis* – The dried *Saururus chinensis* (1 kg) were extracted with methanol (MeOH) at room temperature for 7 days. The extract solutions were filtered and then evaporated at 40 °C under reduced pressure. The MeOH extract (ME; 113 g) were resuspended in 500 ml water and then methylene chloride (CH_2Cl_2), ethylacetate (EtOAc) and *n*-butanol (BuOH), successively to give fractions, CH_2Cl_2 fraction (MF; 61 g), EtOAc fraction (EF; 1.2 g), BuOH fraction (BF; 12.5 g) and water fraction (WF; 28 g), respectively.

Cell culture and induction of multinucleated osteoclasts – All materials for cell culture were purchased from HyClone (UT). Osteoclast generation was achieved using mouse bone marrow-derived macrophages (BMMs). For the generation of bone marrow-derived osteoclasts, monocytes were isolated from femur and tibiae of BALB/c mice (Central Lab. Animal Inc., Korea), seeded and cultured in a-MEM with 10% Fetal bovine serum (FBS) and 10 ng/ml macrophage colony stimulating factor (M-CSF; R&D Systems Inc.) for 1 day. Suspended cells at this stage were considered M-CSF-dependent BMMs and used as osteoclasts precursors. Induction of differentiation to osteoclasts was achieved by culturing those cells plated into a 96-well plate at the density of 3×10^5 cells/well in aMEM with 10% FBS, 100 ng/ml RANKL and 30 ng/ml M-CSF. Multinucleated osteoclasts were observed on the differentiation day 6.

TRAP staining and activity assay – Multinucleated osteoclasts were fixed with 10% formalin for 10 min and ethanol/acetone (1 : 1) for 1 min, and then stained by Leukocyte Acid Phosphatase Kit 387-A (Sigma, MO). The images of TRAP-positive multinucleated cells were captured under a microscope with DP Controller (Olympus Optical, Japan). For measuring TRAP activity, multinucleated osteoclasts were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, and then 100 μl of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate (Sigma, MO) was added to the dried cells. After incubation for 1 h, the enzyme reaction mixtures in the wells were transferred into new plates containing an equal volume of 0.1 N NaOH. Absorbance was measured at 410 nm, and TRAP activity was presented as % of control. The experiment was performed in triplicate and differences were considered significant when $P < 0.05$.

Cell viability assay – BMMs were suspended in a-MEM with 10% FBS, and plated in 96-well plates at the density 3×10^5 cells/well. After 24 h, serially diluted extracts were treated and incubated for 3 days. Cell viability was then evaluated by Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol. The experiment was performed in triplicate.

Western blot analysis – Cells were homogenized in ice-cold protein extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, and one protease inhibitor cocktail tablet (Roche, Germany) and centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was used as cytoplasmic protein fraction and nuclear proteins were extracted by NucBuster Protein Extraction kit (Novagen, Germany). The protein concentration was determined by BCA protein assay kit (Pierce, IL). Protein samples (10 μg) were mixed with sample buffer (100 mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, 0.01% bromopenol blue, pH 7.6), incubated at 100 °C for 5 min, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using Mini protean 3 Cell (Bio-Rad, CA). Proteins separated on the gels were transferred onto nitrocellulose membrane (Whatman, Germany) and the transferred membranes were stained with Ponceau S staining solution in order to ascertain the loading amount of proteins and the efficiency of transfer. The stained membrane was washed and incubated in blocking buffer

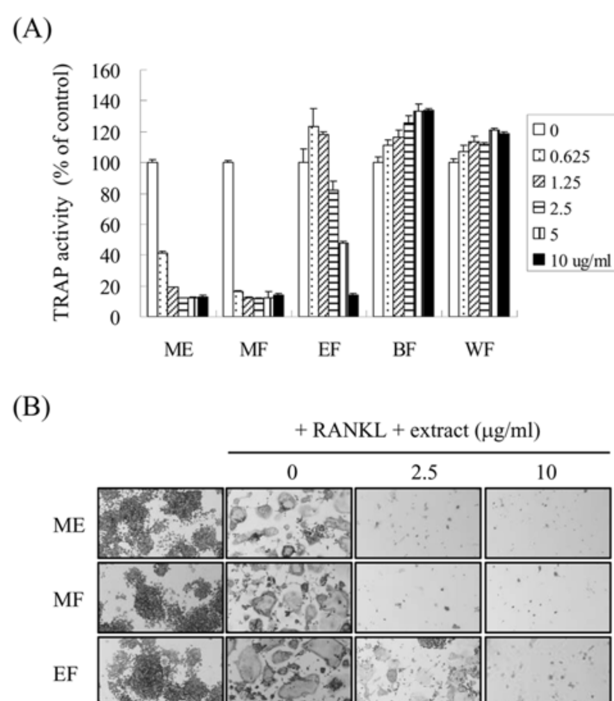

Fig. 1. Effect of *Saururus chinensis* extracts on the RANKL-induced TRAP activity (A), formation of TRAP-positive multinucleated osteoclasts (B) in RAW264.7 cells. The generation of multinucleated osteoclasts in RAW264.7 cells was achieved as described in 'Materials and Methods'.

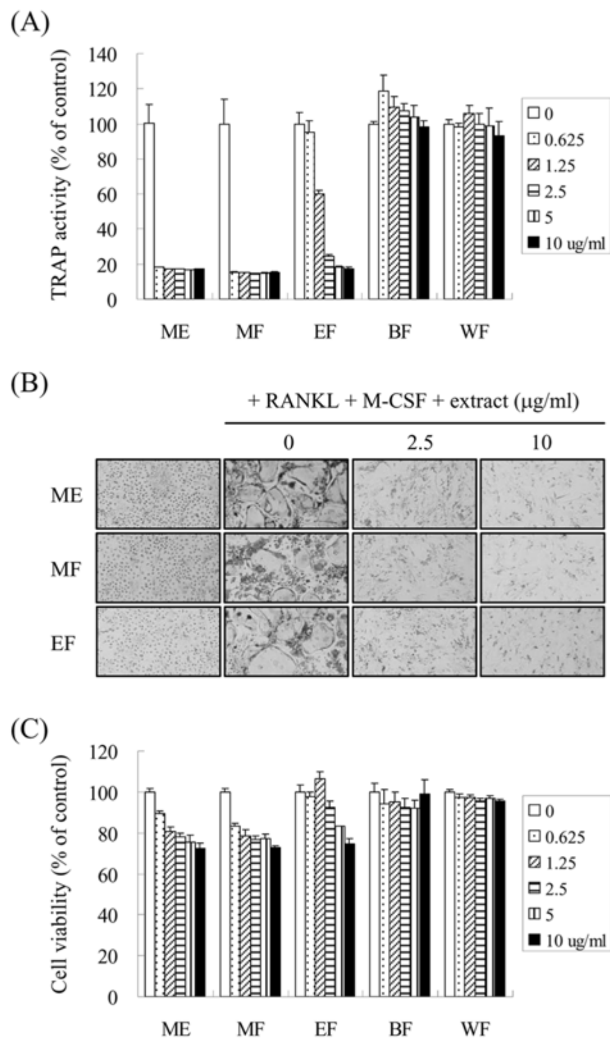

Fig. 2. Effect of *Saururus chinensis* extracts on the RANKL/M-CSF-induced TRAP activity (A), formation of TRAP-positive multinucleated osteoclasts (B) in BMMs. The generation of multinucleated osteoclasts in BMMs was achieved as described in 'Materials and Methods'. (C) The effect of extracts on cell viability was evaluated as described in 'Materials and Methods'.

(10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 3% nonfat dry milk). The membrane was then incubated for 2 h at room temperature with 1 : 1000 diluted primary antibody (Santa Cruz Biotechnology, CA). After washing with blocking buffer three times for 15 min, membrane was probed with 1 : 2000 diluted secondary antibody for 2 h. The membrane was then washed three times for 15 min and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, IL). Chemiluminescent signal was detected with LAS-3000 Luminescent image analyzer (Fuji Photo Film Co., Japan).

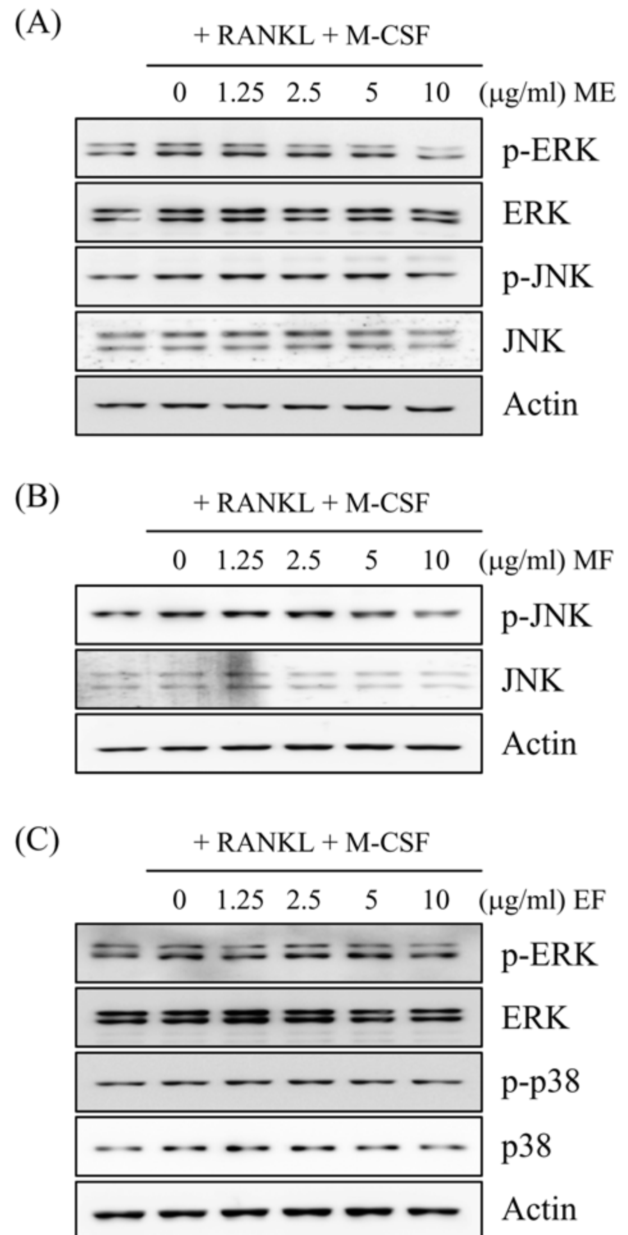

Fig. 3. Effects of *Saururus chinensis* extracts ME (A), MF (B) and EF (C) on activation of MAP kinases in BMMs. The generation of multinucleated osteoclasts in BMMs was achieved as described in 'Materials and Methods'. For Western blot analysis, M-CSF-dependent BMMs were plated in a 60-mm dish at the density of 4×10^6 cells. After 1 day incubation, *Saururus chinensis* extracts was treated into cells 2 h before treatment of both RANKL and M-CSF and protein samples were prepared 1 h after treatment of both RANKL and M-CSF. Actin was used as an internal control in cytosol fractions, respectively.

Results

In order to evaluate the activity of *Saururus chinensis* extracts in osteoclasts differentiation, two *in vitro* models

were used. As shown in Fig. 1, three out of five extracts from *Saururus chinensis* dramatically inhibited TRAP activity in RAW264.7 cells. ME, MF and EF inhibited the formation of multinucleated osteoclasts. However, BF and WF did not show any effect on osteoclast differentiation. The inhibitory effects of ME, MF, and EF on TRAP activity and multinucleated osteoclast formation in RAW264.7 cells were consistent with those in BMMs (Fig. 2). The IC_{50} values for TRAP activity in BMMs were 0.38 ± 0.04 μg/ml for ME, 0.37 ± 0.04 μg/ml for MF and 1.59 ± 0.07 μg/ml for EF, respectively. Considering that extracts with inhibitory activity in osteoclast formation were not shown to strongly decrease the cell viability of BMMs, the inhibitory effects of them might not be related with their cytotoxicity; for example, ME at 0.625 μg/ml showed 20% TRAP activity with 90% cell viability of control.

To elucidate the action mechanism of *Saururus chinensis* extracts in the process of osteoclast differentiation, the effects of them on the activation of MAP kinases in BMMs were evaluated by Western blot analysis. Interestingly, ME inhibited the phosphorylation of the extracellular signal-regulated kinase (ERK) in a dose-dependent manner (Fig. 2A). At 2.5 μg/ml, ME, MF and EF inhibited TRAP activity and formation of multinucleated osteoclasts, but they failed to inhibit the activation of MAP kinase. However, at 10 ug/ml, ME and MF were shown to inhibit the phosphorylation of c-jun-N-terminal kinase (JNK) (Fig. 2A and 2B) and EF inhibited the phosphorylation of ERK and p38 (Fig. 2C).

Discussion

In this study, we found that ME, MF and EF isolated from *Saururus chinensis* have the potential to inhibit the osteoclast differentiation and their activities result from the inhibition of MAP kinases activations. Interestingly, ME inhibited the phosphorylation of ERK in a dose-dependent manner and at 10 μg/ml, it also inhibited that of JNK. MF and EF failed to inhibit the activation of MAP kinase at low concentrations, but at high concentrations, they were shown to slightly inhibit MAP kinase activation in part.

The activation of MAP kinases through RANK signaling has been well characterized in osteoclast differentiation. ERK has a role on osteoclast survival by preventing spontaneous apoptosis without affecting on the bone-resorbing activity of osteoclast (Miyazaki *et al*., 2000). p38 directly links to generate osteoclasts by either co-culturing with osteoblasts or by adding soluble RANKL, which reveal a pivotal role of p38 activation in osteoclast differentiation (Matsumoto *et al*., 2000). Recently, it has suggested that JNK activation is required for efficient osteoclast differentiation from bone marrow monocytes, and to protect bone marrow monocytes from RANKL-induced apoptosis during osteoclast differentiation (David *et al*., 2002).

Isolated components of herb have shown an inhibitory effect on osteoclastogenesis by inhibiting the activation of MAP kinase signaling pathway. Sauchinone from *n*-hexane fraction of *Saururus chinensis* greatly attenuated the activation of ERK and p38, but not JNK in response to RANKL, inhibiting osteoclast differentiation and bone resorption (Han *et al*., 2007). 25-Acetylcimigenol xylopyranoside isolated from ethyl acetate partition of black cohosh abrogated RANKL-induced activation of ERK and NF-κB, less significantly on p38 and JNK, which repressed the bone resorptive activity of differentiated osteoclasts (Qiu *et al*., 2007). Furthermore, tanshinone IIA extracted from methylene chloride fraction of *Salvia miltiorrhiza* Bunge suppressed ERK, Akt and NF-κB activation but increase p38 activation in osteoclast precursor cells (Kim *et al*., 2004). In this regard, the inhibitory effects of natural components from different herbs on osteoclast differentiation would be mainly associated with the disturbance in MAP kinase signal transduction. However, considering that MF and EF strongly inhibited osteoclast differentiation, but failed to inhibit the activation of MAP kinase at low concentrations, there might be other signaling molecules to be inhibited by both extracts.

Recently, sauchinone and saucerneol, lignans isolated from *n*-hexane or methylene chloride fraction of *Saururus chinensis*, have been reported to inhibit osteoclast differentiation and bone resorption (Han *et al*., 2007, Kim *et al*., in press). In a previous study, we also found that one more compounds isolated from *Saururus chinensis* inhibited osteoclastogenesis (unpublished data). The inhibitory activities of these compounds in osteoclast differentiation might be related to those of extracts of *Saururus chinensis*.

In conclusion, ME, MF and EF, not BF and WF, in *Saururus chinensis* extracts have the potential to inhibit the osteoclast differentiation suggesting the possible usefulness of them for the treatment of bone resorption-related diseases. Since we have the experimental evidence for the inhibitory activities of *Saururus chinensis* extracts in ovariectomized rat model (unpublished data), further studies should be required to determine the precise mechanism and biological efficacy of *Saururus chinensis*

extracts *in vivo* model and identify bioactive small molecules with the potential to inhibit osteoclast differentiation in those fractions.

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