INTRODUCTION:
Numerous studies have indicated that inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with osteoporosis. D-pinitol (3-O-methyl-chiroinositol), an active component of the traditional antidiabetic plant, Bougainvillea spectabilis, reportedly exerts insulin-like effects. In addition, D-pinitol has been suggested to possess multifunctional properties, including anti-inflammatory activity. Here we found that D-pinitol markedly inhibited the receptor activator of nuclear factor kappa B ligand (RANKL) induced osteoclastic differentiation from bone marrow stromal cells and RAW264.7 macrophage cells. Treatment of RAW264.7 macrophages with RANKL induced p38 and c-Jun N-terminal kinase (JNK) phosphorylation. RANKL-induced p38 and JNK phosphorylation was attenuated by D-pinitol. Furthermore, RANKL-mediated increase of IKKα/β, IkBα and p65 phosphorylation at Ser536, NF-κB-luciferase activity and NF-κB binding activity was also inhibited by D-pinitol. Our data suggest that D-pinitol inhibits osteoclastogenesis from bone marrow stromal cells and macrophage cells via attenuated of RANKL-induced p38, JNK and NF-κB activation.

METHODS:
Cell culture: Bone marrow cells were prepared by removing from femurs of 6–8 week-old Sprague–Dawley rats and flushing the bone marrow cavity with α-MEM which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). The non-adherent cells (hematopoietic cells) were collected after 24 h and used as osteoclast precursors. Murine RAW264.7 cells (a mouse macrophage cell line obtained from American Type Culture Collection) were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For differentiation of osteoclasts, Hematopoietic cells and RAW264.7 cells were seeded at a density of 2×10^4 cells/well, in 24-well plate, then cultured in the presence of RANKL (50 ng/ml) for 5 days. The culture medium was replaced every 3 days.

Osteoclast differentiation assay: Osteoclast formation was measured by quantifying cells positively stained by tartrate-resistant acid phosphatase [TRAP (Acid Phosphatase Kit 387-A; Sigma-Aldrich, St. Louis, MO, USA)]. Briefly, the cells were fixed for 30 second and then stained with Naphthol AS-BI phosphate and a tartrate solution for 1 h at 37 °C, followed by counterstaining with a hematoxylin solution. Osteoclasts were determined to be TRAP-positive staining multinuclear (>3 nuclei) cells using light microscopy. The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted. The morphological features of osteoclasts were also photographed.

Western blot analysis; Transfection and reporter gene assay
EMS assay

RESULTS:
Osteoclasts are specialized monocyte/macrophage family members that differentiate from bone marrow hematopoietic precursors. Cultures of osteoclast precursors in the presence of RANKL (50 ng/ml) for 5 days induced the formation of large mature osteoclasts with multi-nuclei characterized by the acquisition of mature phenotypic markers, such as TRAP (Fig. 1A). Treatment with D-pinitol (10 μM) markedly inhibited the differentiation of osteoclast (Fig. 1A and 1C; left panel). The stimulating effect on osteoclast differentiation was also observed in murine RAW264.7 macrophages, where RANKL (50 ng/ml) were able to cause osteoclast formation. Culturing for 5 days in RAW264.7 cells, D-pinitol(3 μM and 10 μM) inhibited the formation of TRAP-positive cells (Fig. 1C; right panel).

These data indicated that D-pinitol inhibited osteoclastogenesis. It has been reported that p38 and JNK mediated RANKL-induced osteoclast formation. Pretreatment of RAW264.7 cells for 30 min with D-pinitol (3 and 10 μg/ml) markedly inhibited the RANKL (50 ng/ml) induced the phosphorylation of p38 and JNK(see Fig. 2A and 2B) and activity(Fig. 2C and 2D). In addition to MAPKs, activation of transcription factor NF-κB is also involved in osteoclast differentiation. Treatment of cells with D-pinitol reduced RANKL-induced IKK, IkBα and p65 phosphorylation, NF-κB luciferase activity and NF-κB binding activity (Fig. 3).

DISCUSSION:
The present study demonstrated that D-pinitol inhibits the osteoclastogenesis either from bone marrow stromal cells or from macrophages. In addition, D-pinitol attenuated RANKL-induced p38, JNK and NF-κB activation. However D-pinitol did not affect the cell proliferation and differentiation of human osteoblasts. Therefore, D-pinitol may show potential beneficial effects by reducing osteoclast formation to benefit bone health.