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Screening for natural medicines effective for the treatment of osteoporosis

Ryuichiro Suzuki[1]*, Satoshi Fukami[1], Mineko Tomomura[2, 3], Akito Tomomura[3] and Yoshiaki Shirataki[1]

[1] Department of Pharmaceutical Sciences, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University

[2] Integrated Education Center, Meikai University

[3] Division of Biochemistry, Department of Oral Biology & Engineering, Meikai University School of Dentistry

* Correspondence to: e-mail: ryu_suzu@josai.ac.jp, Department of Pharmaceutical Sciences, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

Abstract

Bone-forming osteoblasts are differentiated from mesenchymal stem cells and dysregulation of this differentiation can lead to osteoporosis. Meanwhile, bone-resorbing osteoclasts are both differentiated and multinucleated from hematopoietic precursor cells of monocyte and/or macrophage lineage. Bone resorption inhibitors such as bisphosphonates and estrogen are used to treat osteoporosis. However, the adverse effects of the long-term use of these medicines are of concern. Therefore, the development of new therapies to ameliorate osteoporosis is desired. Therefore, in the present study, we screened 22 plant extracts and found that nine methanolic extracts of medicinal plants promote the differentiation of MC3T3-E1 cells to osteoblasts. These nine extracts were then evaluated for their inhibitory activity on osteoclast differentiation in RAW264.7 mouse macrophage cells. Among the nine extracts, *Daucus carota*, *Vitis* spp., *Sasa veitchii*, *Euptelea polyandra*, and *Sesamum indicum* exhibited pro-osteoblastic and anti-osteoclastic activity with low cytotoxicity, suggesting their potential effectiveness against osteoporosis.

Keywords: Medicinal plant extracts, osteoporosis, osteoblast differentiation, osteoclast differentiation, MC3T3-E1 cells, RAW264.7 mouse macrophage cells

Introduction

Osteoporosis is a condition characterized by a decrease in bone density and strength, resulting in fragile bones [1]. Normal bone is dependent on the balance of bone formation and resorption. Postmenopausal osteoporosis is a common bone disease in elderly women. Estrogen deficiency is known to increase bone turnover, leading to osteoclastic bone resorption [2]. For treatment against osteoporosis, bone resorption inhibitors, such as bisphosphonate, calcitonin, and estrogen, and bone formation promoters, such as parathyroid hormone, are used in the clinical setting. However, the adverse effects resulting from the long-term use of these medicines are of concern. For example, estrogen may increase the risk of breast and endometrial cancers [3]. Therefore, the development of alternative medicines against osteoporosis is desired.

In the present study, we screened 22 medicinal plant extracts for the promotion of osteoblast differentiation and mineralization in the osteoblastic cell line MC3T3-E1 and the inhibition of osteoclast differentiation in RAW264.7 mouse macrophage cells.

Materials and methods

Materials

Odontioda Marie Noel ‘Velano’ (leaf: 1, bulb: 2, root: 3), *Odontoglossum* Harvengtense

‘tutu’ (leaf: 4, bulb: 5, root: 6), *Pandanus amaryllifolius* (7), *Daucus carota* (8), *Myrica rubra* (twig: 9, leaf: 10), *Vitis* spp. (11), *Camptotheca acuminata* (12), *Rhinacanthus nasutus* (13), *Sasa veitchii* (14), *Euptelea polyandra* (15), *Nandina domestica* (16), *Theobroma cacao* (17), *Phellodendron amurense* (18), *Sophora flavescens* (cultivated in Hangzhou: 19, cultivated in Beijing: 21), *Actinidia polygama* (20), *Sesamum indicum* (22) were used in this study (Table 1). Voucher specimens were deposited in the Laboratory of Pharmacognosy and Natural Medicines.

Preparation of extracts

Dried samples were extracted with methanol in reflux for an hour three times to give the corresponding extract. These extracts were then evaporated to remove the organic solvent. The resulting extracts were stored at –20°C before use.

Cell culture and osteoblast differentiation

Osteoblastic MC3T3-E1 cells (mouse calvarial origin) were grown in α-minimal essential medium (α-MEM; Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS; Thermo, Melbourne, Australia), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, New York, NY, USA) at 37°C in a

humidified atmosphere of 5% CO_2 in air.

For osteoblast differentiation, after the cells were grown to confluence, the culture medium was changed to an osteogenic medium containing 100 μg/mL ascorbic acid and 100 mM β-glycerophosphate (defined as differentiation medium) with or without samples. The medium was changed every 3 days. The differentiation of osteoblasts was evaluated based on staining alkaline phosphatase (ALP) activity. Cells were then rinsed and fixed with 4% paraformaldehyde. After treatment with ethanol/acetone (1:1) mixture, ALP activity in the cells was stained using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in 0.5 M Tris buffered saline (pH 9.5). To quantify the ALP staining intensity, stained cells were dissolved in solvent containing 10% sodium dodecyl sulfate (SDS), 50% *N*, *N*-dimethylformamide (DMF) and 10% acetic acid, and measured at 540 nm using a microplate reader.

Cell viability

Cell proliferation and cytotoxicity were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. First, 1 mg/mL MTT solution was added to the cells and incubated for 2 h. After removal of the culture supernatants, formazan crystals were dissolved in isopropanol: dimethyl

sulfoxide (DMSO) (1:1) solution. Absorbance was measured at 595 nm using a microplate reader.

Mineralization assay

Mineral depositions of osteoblasts were measured using Alizarin red S (Cosmo Bio, Tokyo, Japan) and von Kossa staining (Wako Pure Chemical Industries, Osaka, Japan). At the end of culture, cells were fixed with 4% paraformaldehyde and stained with 40 mM Alizarin red S (pH 6.4) for 10 min. Fixed cells were also stained with von Kossa according to the manufacturer's instructions. Stained cells were then visualized using light microscopy and photographed.

Assay for osteoclast differentiation

Osteoclasts were differentiated from RAW264.7 mouse macrophage cells as described previously [4]. Briefly, cells were cultured with α-MEM containing 10% FBS on 96-well plates. For osteoclast differentiation, the cells were cultured in the presence of the receptor activator of NFκB ligand (RANKL, 10 ng/mL; R & D Systems, Minneapolis, MN, USA) with or without test samples for 4 days. Next, the tartrate-resistant acid phosphatase (TRAP) activity of the medium was measured and

TRAP staining was performed.

Measurement of TRAP activity and TRAP staining

The TRAP activity of osteoclasts was measured with culture media (30 μL) incubated for 20 min at 37°C with 30 μL of 600 mM sodium acetate buffer (pH 5.5) containing L-ascorbic acid (17.6 mg/mL), sodium tartrate dehydrate (9.2 mg/mL), 4-nitrophenylphosphate Na (3.6 mg/mL), Triton X-100 (0.3%), ethylenediaminetetraacetic acid (EDTA) (6 mM), and NaCl (600 mM). The reaction was terminated by the addition of 30 μL of NaOH (300 mM), and then the absorbance was measured at 405 nm. TRAP histochemical staining of the cells was performed using a leukocyte acid phosphatase kit (Sigma-Aldrich, St. Louis, MO, USA). Cultured cells were fixed with 100% methanol for 1 min at room temperature and air-dried. TRAP staining was performed according to the manufacturer's instructions. TRAP-positive multinucleated (more than three nuclei) cells were photographed under inverted phase-contrast microscopy [5].

Statistical analysis

The data are presented as mean±standard deviation of at least three independent

experiments. Statistical analysis was performed using the Student's t-test. In all analyses, $P<0.05$ was taken to indicate statistical significance.

Results and discussion

In the present study, we screened 22 medicinal plant extracts for the promotion of osteoblast differentiation and mineralization in MC3T3-E1 cells. Treatment with *D. carota* (leaf) (8), *S. veitchii* (leaf) (14), and *S. flavescens* (Beijing) (21) extracts more than doubled ALP activity compared with the control. *O*. Harvengtense 'Tutu' (leaf) (4), *M. rubra* (twig) (9), *M. rubra* (leaf) (10), *V.* spp. (root) (11), *R. nasutus* (leaf) (13), *E. polyandra* (leaf) (15), and *S. indicum* (leaf) (22) enhanced ALP activity more than 1.5 times more than the control in a dose-dependent manner (Fig. 1a). No stimulation of cell proliferation and cytotoxicity was observed except for *M. rubra* (twig) (9) and *M. rubra* (leaf) (10), and high doses of *O*. Marie Noel 'Velano' (leaf) (1), *O*. Harvengtense 'tutu' (leaf) (4), *E. polyandra* (15), and *S. flavescens* (Beijing) (21) (Fig. 1b). *O*. Marie Noel 'Velano' (bulb) (2), *O*. Marie Noel 'Velano' (root) (3), *O*. Harvengtense 'Tutu' (root) (6), *C. acuminata* (leaf) (12), and *N. domestica* (leaf) (16) decreased ALP and MTT activity at concentrations of 50 μg/mL (Fig. 1a). However, these extracts did not enhance ALP activity at any concentration, including less than 5 μg/mL (data not

shown). Treatment with a higher concentration (100 μg/mL) of the 17 other extracts, except *S. flavescens* (Hangzhou) (19), showed decreased ALP activity compared with 50 μg/mL (data not shown). Next, based on the results of the first screening, nine medicinal plant extracts (*O.* Harvengtense 'Tutu' (leaf), *D. carota* (leaf), *M. rubra* (twig), *M. rubra* (leaf), *V.* spp. (root), *S. veitchii* (leaf), *E. polyandra* (leaf), *S. flavescens* (root), and *S. indicum* (leaf)) were selected to further confirm the dose-dependent effect on the enhancement of ALP activity. All samples dose-dependently increased ALP activity, reaching the maximum in the range of 25–100 μg/mL (Fig. 2a). *O.* Harvengtense 'Tutu' (leaf) (4) and *E. polyandra* (leaf) (15) inhibited both ALP and MTT activity at a concentration of 100 μg/mL (Fig. 2b). All other extracts except *M. rubra* (leaf) (10) increased ALP activity without inducing cytotoxicity.

Next, the selected plant extracts were evaluated for mineralization activities using Alizarin red S and von Kossa staining (Fig. 3). Calcium deposition was not detected in undifferentiated MC3T3-E1 cells, whereas it was detected in the cells cultured in osteogenic medium for 2 weeks. Treatment of 50 μg/mL of these plant extracts except *S. flavescens* (root) further promoted calcium deposition as evaluated by Alizarin red S staining (Fig. 3). Among these extracts, *M. rubra* (twig) (9), *M. rubra* (leaf) (10), *S. veitchii* (leaf) (14), and *S. indicum* (leaf) (22) promoted marked mineralization of

MC3T3 cells compared with bone morphogenetic protein (BMP). After 3 weeks in culture, mineralization of MC3T3 cells treated with all nine medicinal plant extracts described above was observed by von Kossa staining.

We also investigated whether these nine medicinal plant extracts could inhibit RANKL-stimulated osteoclast formation from RAW264.7 cells. As shown in Fig. 4a, all plant extracts except *S. flavescens* (Hangzhou) (19) dose-dependently inhibited RANKL-induced TRAP activity. *S. flavescens* (Hangzhou) (19) had little effect on RANKL-induced TRAP activity. It should be noted that *Vitis* spp. (root) (11) inhibited TRAP activity without inducing cytotoxicity (Fig. 4b). Although *D. carota* (leaf) (8) and *S. indicum* (leaf) (22) induced mild cytotoxicity, these extracts inhibited TRAP activity. TRAP-positive multinucleated giant cells were observed in the culture treated with RANKL, while treatment with all extracts except *S. flavescens* (Hangzhou) (19) dose-dependently inhibited RANKL-stimulated multinucleated cell formation in varying degrees (Fig. 5). In addition, 100 μg/mL of *O.* Harvengtense ‘Tutu’ (leaf) (4), *S. veitchii* (leaf) (14), and *E. polyandra* (leaf) (15) completely inhibited RANKL-induced multinucleated mature osteoclasts.

Conclusions

In the present study, we screened 22 medicinal plant extracts for the promotion of osteoblast differentiation and mineralization in MC3T3-E1 cells. Nine extracts showed enhanced ALP activity and mineralization in MC3T3-E1 cells. Furthermore, the inhibitory effects of these candidates on osteoclast differentiation were evaluated. The results suggest that *D. carota* (8), *V.* spp. (11), *S. veitchii* (leaf) (14), *E. polyandra* (15), and *S. indicum* (22) have potential as effective medicinal plant extracts against osteoporosis because they promote osteoblast differentiation while inhibiting osteoclast differentiation without cell damage. In a future study, we plan to identify the active components of these medicinal plants and clarify their underlying mechanisms.

References

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Figure legends

Fig. 1 ALP activity in MC3T3-E1 cells treated with various medical plant extracts. (**a**) MC3T3-E1 cells were cultured either in undifferentiated media or in differentiated media untreated (control) or treated with the indicated concentration of plant extracts for 4 days. Cells were stained for ALP activity, solubilized, and then measured for absorbance. The ALP activities are expressed as a ratio to control culture. (**b**) Cell toxicity of MC3T3-E1 cells was evaluated by MTT assay. Cells were cultured as shown in Fig 1a. MTT assay was performed as described in the "Materials and Methods". Cell viabilities are expressed as a ratio to control culture. Each column represents the mean±SD of three independent experiments. *P<0.05 vs. control.

Fig. 2 ALP activity in MC3T3-E1 cells treated with selected medical plant extracts. (**a**) MC3T3-E1 cells were cultured as shown in Fig. 1a with or without selected medical plant extracts. ALP activities are expressed as a ratio to control culture. (**b**) Cell toxicity of MC3T3-E1 cells was evaluated by MTT assay as shown in Fig. 1b. Each column represents the mean±SD of three independent experiments. *P<0.05 vs. control.

Fig. 3 Mineral deposition in MC3T3-E1 cells treated with selected medical plant

extracts. MC3T3-E1 cells were cultured as shown in Fig. 1a for the indicated times. The cells were stained with Alizarin red S and von Kossa to measure mineral deposition. BMP was used as a positive control for osteoblast differentiation.

Fig. 4 TRAP activity in RAW264.7 cells treated with selected medical plant extracts. (**a**) RAW264.7 cells were cultured in either undifferentiated media or RANKL-treated differentiated media without (control) or with selected medical plant extracts for 4 days. TRAP activity in the culture medium was measured (**a**) and MTT assay (**b**) was performed, as described in the “Materials and Methods”. Each column represents the mean±SD from three independent experiments. *$P<0.05$ vs. control.

Fig. 5 TRAP-positive multinucleated cell formation from RAW264.7 cells treated with selected medical plant extracts. RAW264.7 cells were cultured as shown in Fig. 4. Cells were stained for TRAP activity as described in the “Materials and Methods”.

Table 1. Medicinal plants evaluated against osteoporosis.

No.	Scientific name	Part
1	*Odontioda* Marie Noel 'Velano’	leaf
2	*Odontioda* Marie Noel 'Velano’	bulb
3	*Odontioda* Marie Noel 'Velano’	root
4	*Odontoglossum* Harvengtense ‘Tutu’	leaf
5	*Odontoglossum* Harvengtense ‘Tutu’	bulb
6	*Odontoglossum* Harvengtense ‘Tutu’	root
7	*Pandanus amaryllifolius*	leaf
8	*Daucus carota*	leaf
9	*Myrica rubra*	twig
10	*Myrica rubra*	leaf
11	*Vitis* spp.	root
12	*Camptotheca acuminata*	leaf
13	*Rhinacanthus nasutus*	leaf
14	*Sasa veitchii*	leaf
15	*Euptelea polyandra*	leaf
16	*Nandia domestica*	leaf
17	*Thobroma cacao*	husk
18	*Phellodendron amurense*	leaf
19	*Sophora flavescens* (Hangzhou)	root
20	*Actinidia polygama*	root
21	*Sophora flavescens* (Beijing)	root
22	*Sesasmum indicum*	leaf

Fig. 1 Suzuki et al

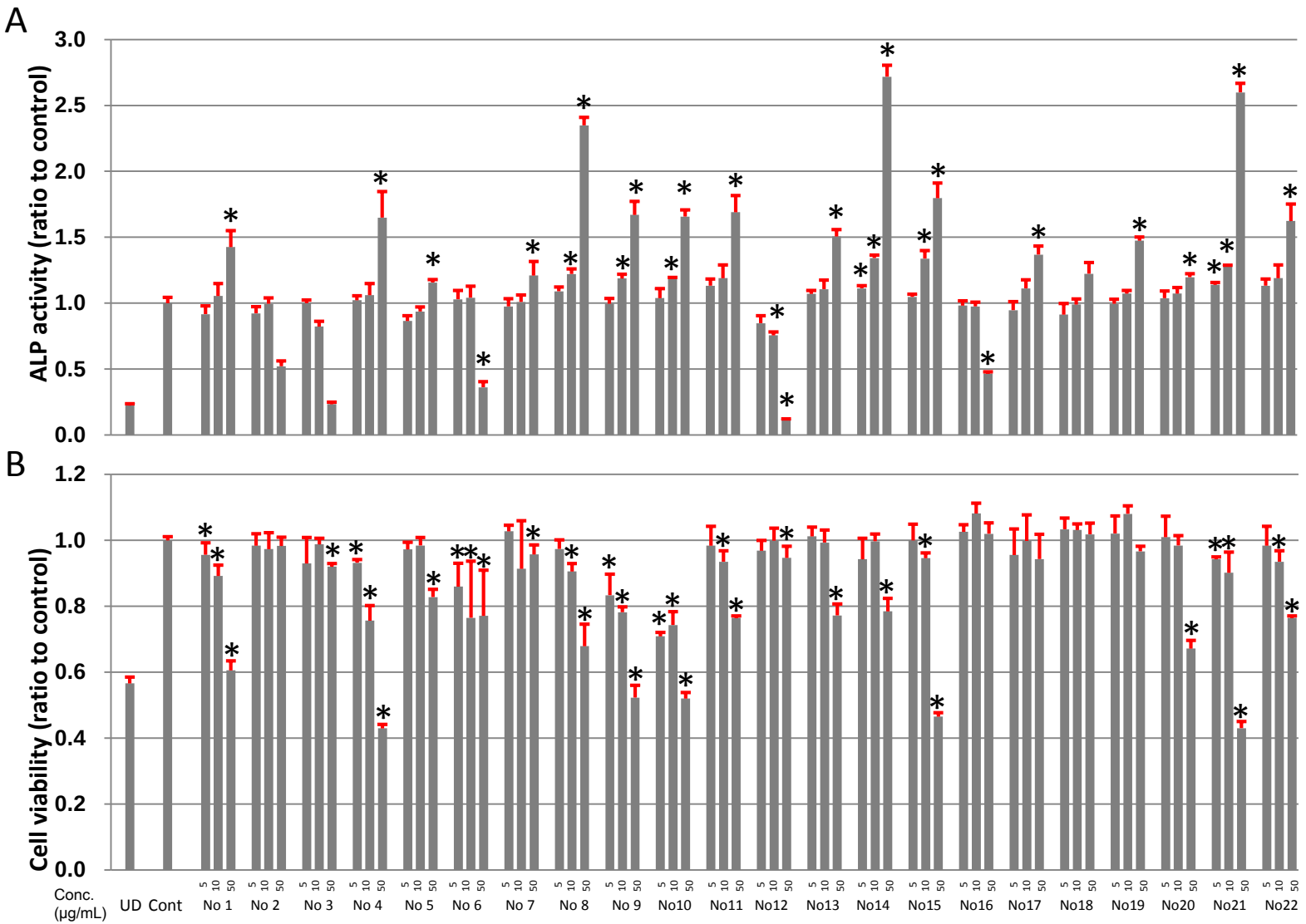

Fig. 2 Suzuki et al

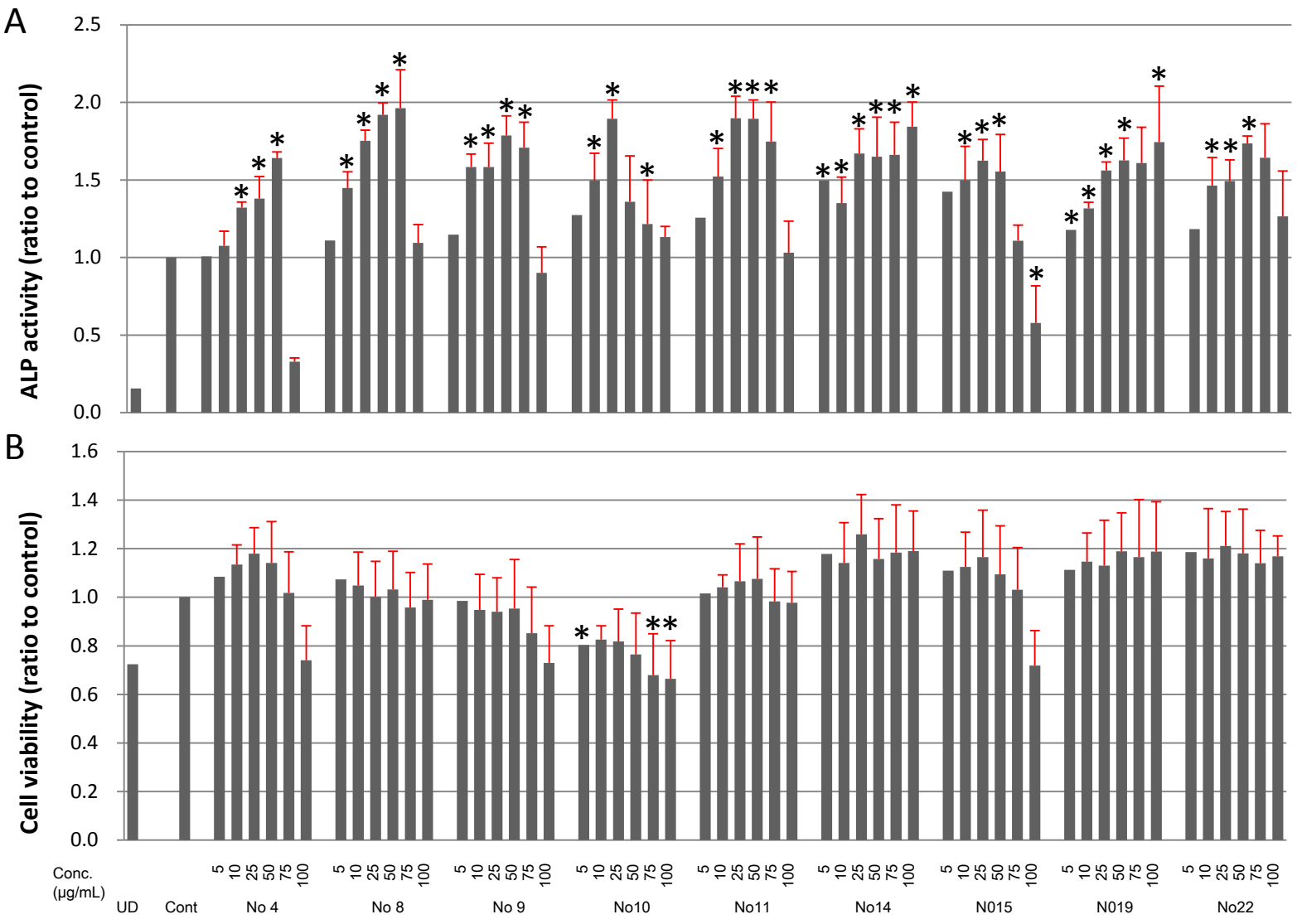

Fig. 3 Suzuki et al

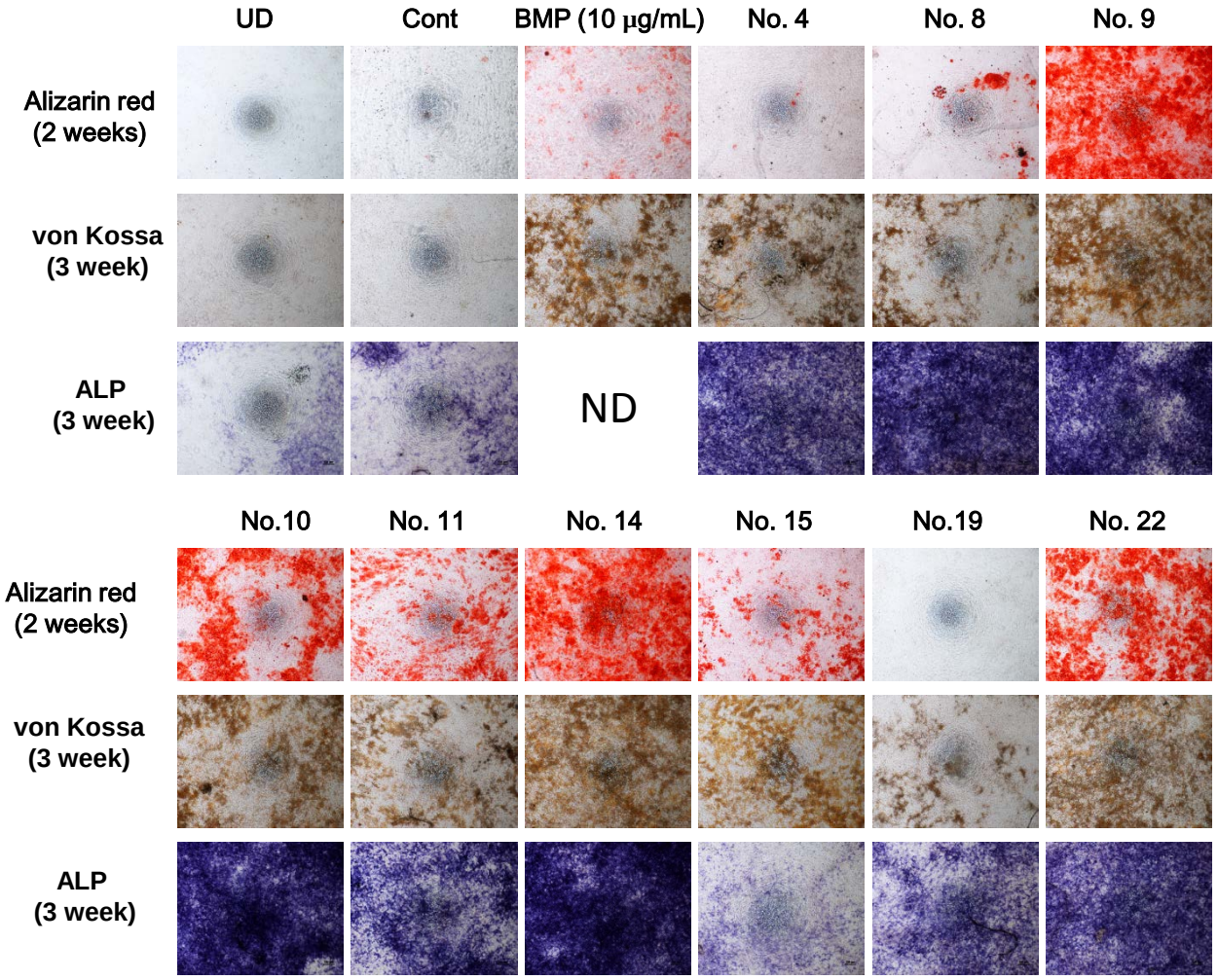

Fig. 4 Suzuki et al

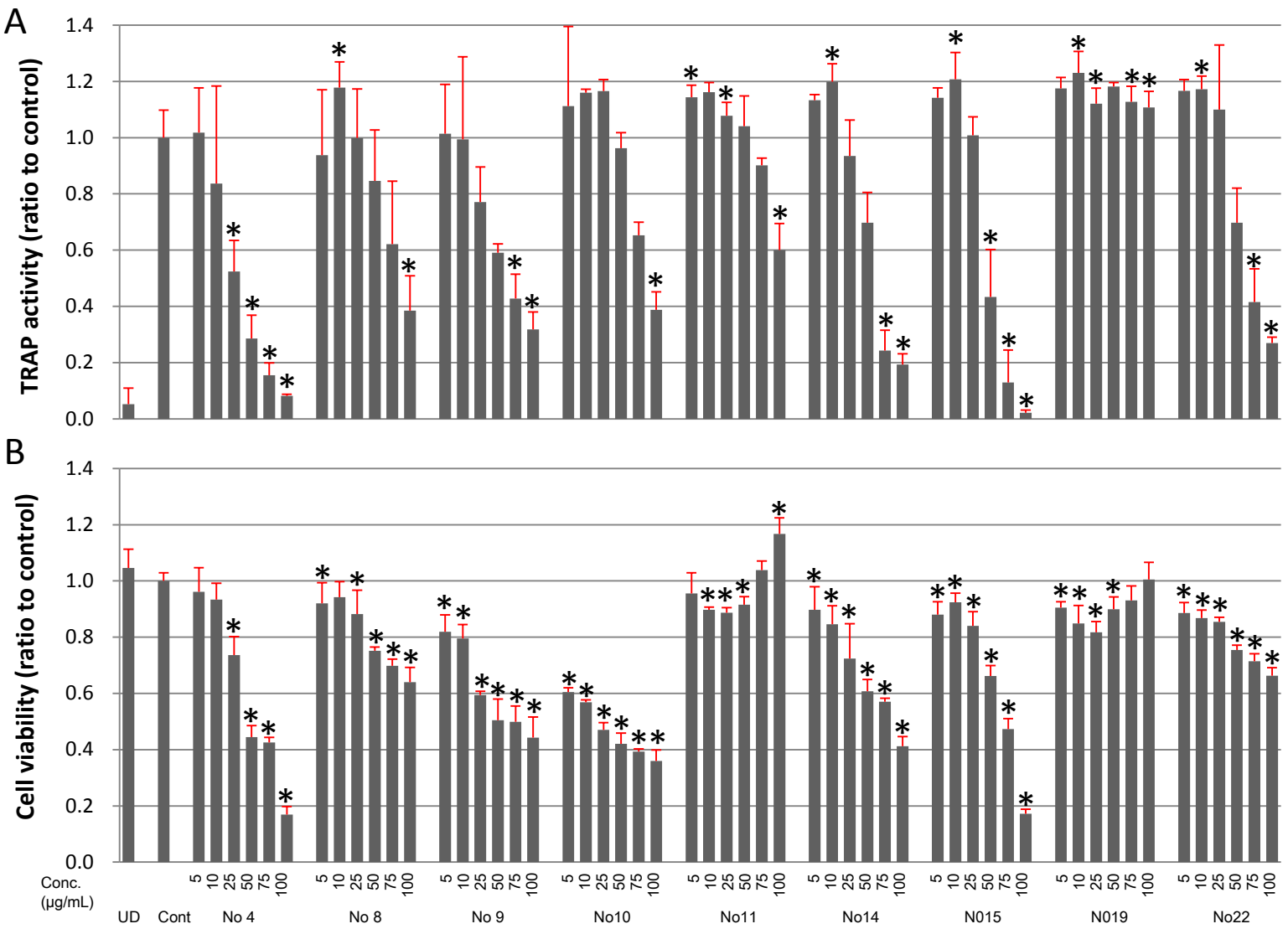

Fig. 5 Suzuki et al