in vivo *25*: 375-380 (2011)

Diverse Biological Activity of *Odontioda* Marie Noel 'Velano' Extracts

YUKO MASUDA[1], JUN-YA UEDA[1], MASAFUMI TAMURA[1], HIROSHI SAKAGAMI[2,3], MINEKO TOMOMURA[3,4], AKITO TOMOMURA[4] and YOSHIAKI SHIRATAKI[1]

[1]*Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan;*
[2]*Division of Pharmacology,* [3]*MPL and* [4]*Division of Biochemistry,*
Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan

Abstract. *Background: Several pharmacologically active substances have been isolated from orchid plants other than Odontioda Marie Noel 'Velano'. Whether or not MeOH extract fractions from O. Marie Noel 'Velano' bulbs exert various biological activities was investigated. Materials and Methods: The MeOH extract was stepwise separated by organic solvents into n-hexane, EtOAc, n-BuOH and H_2O layer fractions. Cytotoxic activity against human tumor and normal cells was determined by the MTT method. Nitric oxide (NO) was determined by the Griess method. Osteoclastogenesis was monitored by tartrate-resistant acid phosphatase (TRAP) activity. Result: The EtOAc fraction showed the highest tumor-specific cytotoxicity, followed by the n-hexane and other fractions. The EtOAc and n-BuOH fractions protected the cells from the cytotoxicity induced by UV irradiation. The EtOAc and n-hexane fractions inhibited NO production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like cells. The EtOAc fraction most strongly inhibited the receptor activator for nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis, followed by the n-BuOH, n-hexane and H_2O fractions. Conclusion: Most of the biological activities tested were concentrated in the EtOAc fraction, and separation from cytotoxic substances is needed to identify the active principle(s).*

Various pharmacologically active substances have been isolated from the stalks of some orchid species (*Prosthechea michuacana*, *Dendrobium nobile*, *Bletilla striata*, *Dendrobium densiflorum*, *Cremastra appendiculata*). These compounds have shown radical-scavenging, antioxidant (1), antifibrosis (2), antitumor (3), antimicrobial (3, 4), and antiplatelet aggregation (5) activities, growth modulation of mouse T and B lymphocytes (6) and antimuscarinic activity (7). However, compounds have not yet been isolated from another species of orchid, *Odontioda* Marie Noel 'Velano' (Figure 1). A study to isolate new compounds from this species was therefore initiated. As a first step, the MeOH extract of *O.* Marie Noel 'Velano' was roughly separated into four fractions by stepwise partitions with organic solvents, and whether or not these separated fractions showed various biological activities, such as tumor-specific cytotoxicity, protection of cells from the cytotoxicity induced by ultraviolet (UV) irradiation, inhibition of nitric oxide (NO) production by lipopolysaccharide (LPS)-activated macrophage-like cells and inhibition of receptor activator for nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis was investigated.

Correspondence to: Prof. Yoshiaki Shirataki, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan. Tel: +81 492717053, Fax: +81 492717984, e-mail: shiratak@josai.ac.jp

Key Words: Odontioda Marie Noel 'Velano' extracts, cytotoxicity, UV protection, macrophage, LPS, RANKL, NO, osteoclastogenesis.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA); fetal bovine serum (FBS) (SAFC Biosciences, St. Louis, MO, USA); RPMI-1640 mediun, α-minimal essential medium (α-MEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS from *Escherichia coli* (serotype 0111:B4) (Sigma-Aldrich, St. Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chemical, Osaka, Japan) and RANKL (R & D Systems, Minneapolis, MN, USA).

Sequential fractionation with organic solvents. Bulbs of *O.* Marie Noel 'Velano' (Orchidaceae) were supplied from Nichirei Garden, Nagano Prefecture, Japan, during the April of 2009. This specimen was proved and identified by Mr. H. Sumiyoshi (Nichirei Garden) and also a voucher specimen (#20090617) is deposited in the Medicinal Plant Garden of Josai University.

The air-dried bulbs of *O.* Marie Noel 'Velano' (63.6 g) were extracted three times with MeOH under reflux for 3 hours. H_2O (80 ml) was added to the MeOH solution (800 ml) which was then partitioned with *n*-hexane (800, 400, 400 ml). The aqueous MeOH layer solution was evaporated, and H_2O (100 ml) was added to the

Figure 1. *Odontioda Marie Noel 'Velano'*.

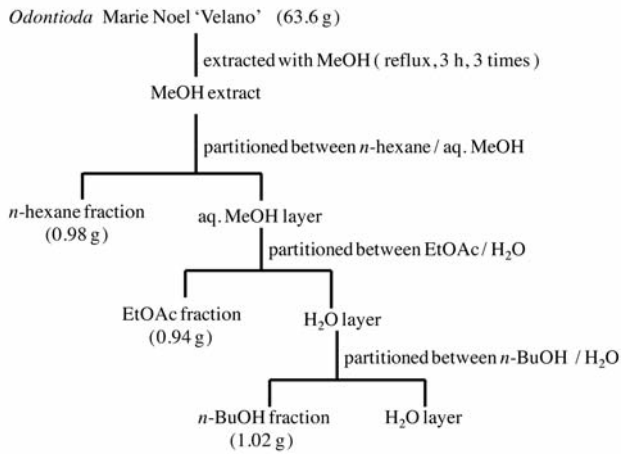

Figure 2. *Sequential fractionation of MeOH with organic solvents*.

residual aqueous solution (100 ml) which was then extracted with EtOAc (200, 100, 100 ml) and *n*-BuOH (100, 50, 50 ml), successively. The *n*-hexane and EtOAc layer solutions were dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo* to yield *n*-hexane (0.98 g) and EtOAc (0.94 g) fractions respectively. The *n*-BuOH and part of the aqueous layer solutions were also evaporated to give *n*-BuOH (1.02 g) and H_2O fractions, respectively (Figure 2).

Cell culture. Human promyelocytic leukemia HL-60 cells were provided by Professor K. Nakaya, Showa University, Japan. Human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4) were provided by Professor M. Nagumo, Showa University. Normal human oral cells, gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF) were prepared from periodontal tissues, according to the guideline of the Intramural Ethic Committee (No. A0808), after obtaining informed consent from a 12-year old patient at the Meikai University Hospital. Since normal oral cells have a limited lifespan of 43-47 population doubling levels (PDL) (8), they were used at 8-15 PDL. Mouse macrophage-like RAW264.7 cells (9) were purchased from Dainippon Sumitomo Pharma, Osaka, Japan. The HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under a humidified 5% CO_2 atmosphere. The other cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate. The normal cells were detached with 0.25% trypsin-0.025% EDTA-2Na in phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS(–)) and subcultured at a 1:4 split ratio once a week, with a medium change in between the subcultures. The tumor cell lines were similarly trypsinized and subcultured.

Assay for cytotoxic activity. The cells (3×10^3 cells/well, 0.1 ml/well) were seeded in 96-microwell plates (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated for 48 hours to allow cell attachment. Near-confluent cells were treated for 48 hours with various concentrations of the test compounds in fresh medium. The relative viable cell number of adherent cells (except for HL-60 cells) was then determined by the MTT method. In brief, control and sample-treated cells were incubated for 4 hours with 0.2 mg/ml of MTT in the culture medium. After removing the medium, the reaction product, formazan, was extracted with DMSO and the absorbance (the relative viable cell number) was measured at 540 nm by a microplate reader (Multiskan Bichromatic Labsystems, Helsinki, Finland). The viability of the suspended cells, *i.e.* HL-60, was determined by cell counting with a hemocytometer after staining with 0.15% trypan blue. The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve. The tumor specificity index (TS) was calculated by the following equation: TS=mean CC_{50} (normal cells)/mean CC_{50} (all tumor cell lines).

Assay of UV protection. HSC-2 cells, that showed the highest sensitivity against UV irradiation among 6 adherent cell lines tested (8), were inoculated into 96-microwell plates (3×10^3 cells/well, 0.1 ml/well) and incubated for 48 hours to allow cell attachment. The culture supernatant was replaced with PBS(–) that contained various concentrations of the test substances, placed at 21 cm distance from a UV lamp (wavelength: 253.7 nm) and exposed to UV irradiation (6 J/m^2/min) for 1 min. The PBS was replaced with fresh DMEM containing 10% FBS, and the cells were incubated for a further 48 hours to determine the relative viable cell number by MTT method, as described above.

Effect on NO production by macrophage-like cells. The RAW264.7 cells (6×10^4 cells/well, 0.1 ml/well) were inoculated into 96-microwell plates and incubated for 3 hours. The medium was then replaced with phenol red-free DMEM containing 10% FBS and the indicated concentrations of the test samples with or without LPS (100 ng/ml). LPS stimulates NO production *via* induction of inducible NO synthase (iNOS) expression (10). After incubation for 24 hours, the NO released into the culture supernatant was measured by the Griess method. From the dose–response curve, the 50% effective concentration (EC_{50}) was calculated. After removing the medium, the attached cells were stained with MTT reagent to

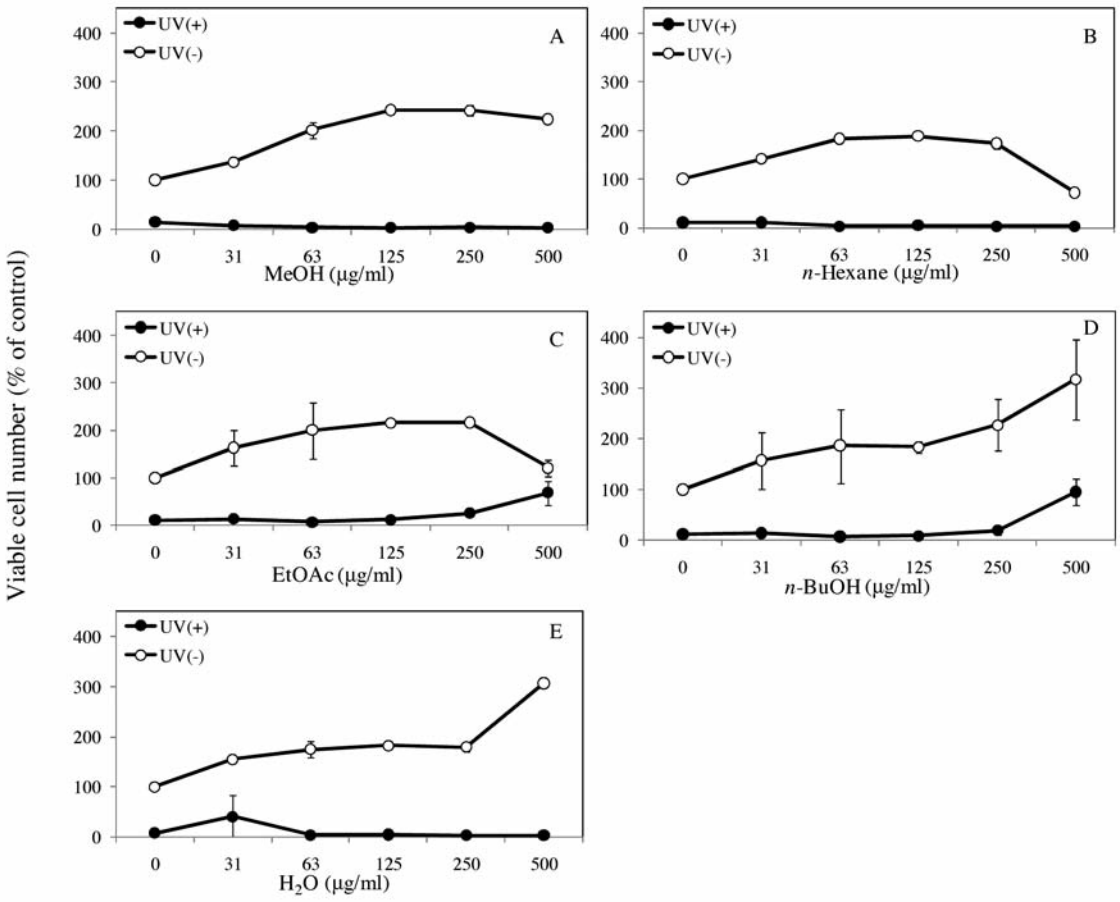

Figure 3. *Effect of O. Marie Noel 'Velano' extracts on UV-induced cytotoxicity. HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 1 min) in PBS(–) containing MeOH extract (A), n-hexane (B), EtOAc (C), n-BuOH (D) or H_2O (E) fraction of the bulb of O. Marie Noel 'Velano'. After 48 hours' incubation in fresh medium, the viable cell number (expressed as a % of control cells, before final incubation that had not been exposed to UV irradiation) was determined. Mean±S.D. of triplicate assays.*

determine the CC_{50}, as described above. The selectivity index (SI) for the inhibition of NO production was determined by the following equation: $SI=CC_{50}/EC_{50}$ (11).

Osteoclastogenesis in cell culture. The RAW264.7 cells were suspended in α-MEM supplemented with 5% FBS and seeded at 2×10^3 cells/well in 96-microwell plates (0.1 ml/well) in the presence or absence of RANKL (10 ng/ml) with or without the test samples. After 4 days in culture, tartrate-resistant acid phosphatase (TRAP) activity of the medium was determined and TRAP staining of the cells was performed.

Measurement of TRAP activity and TRAP-staining. The culture media (30 μl) were incubated for 30 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), disodium 4-nitrophenylphosphate (3.6 mg/ml), Triton X-100 (0.3%), EDTA (6 mM) and NaCl (600 mM). The reaction was terminated by the addition of 30 μl of NaOH (300 mM) and the absorbance at 405 nm was measured by microplate reader. TRAP histochemical staining of the cells was performed using a leukocyte acid phosphatase kit (Sigma-Aldrich). The cultured cells were fixed with 100% MeOH for 1 min at room temperature and air-dried, then stained for TRAP activity.

Statistical analysis. The difference between two groups was evaluated by Student's *t*-test.

Results

Cytotoxicity. The MeOH extract showed the highest cytotoxicity against the HL-60 cells (CC_{50}=106 μM), followed by HSC-4 (CC_{50}=188 μM) > HSC-3 (CC_{50}≥221 μM) > HSC-2 (CC_{50}≥246 μM) (Table I). The human normal oral cells (HGF, HPC, HPLF) showed lower sensitivity to the MeOH extract (CC_{50}>239 μM), with TS of 1.3 (Table I).

Among the four fractions prepared from the MeOH extract (Figure 2), the EtOAc fraction showed the highest cytotoxicity, and the highest tumor-specificity TS 2.1, followed by the *n*-hexane (TS=1.5) > *n*-BuOH and H_2O

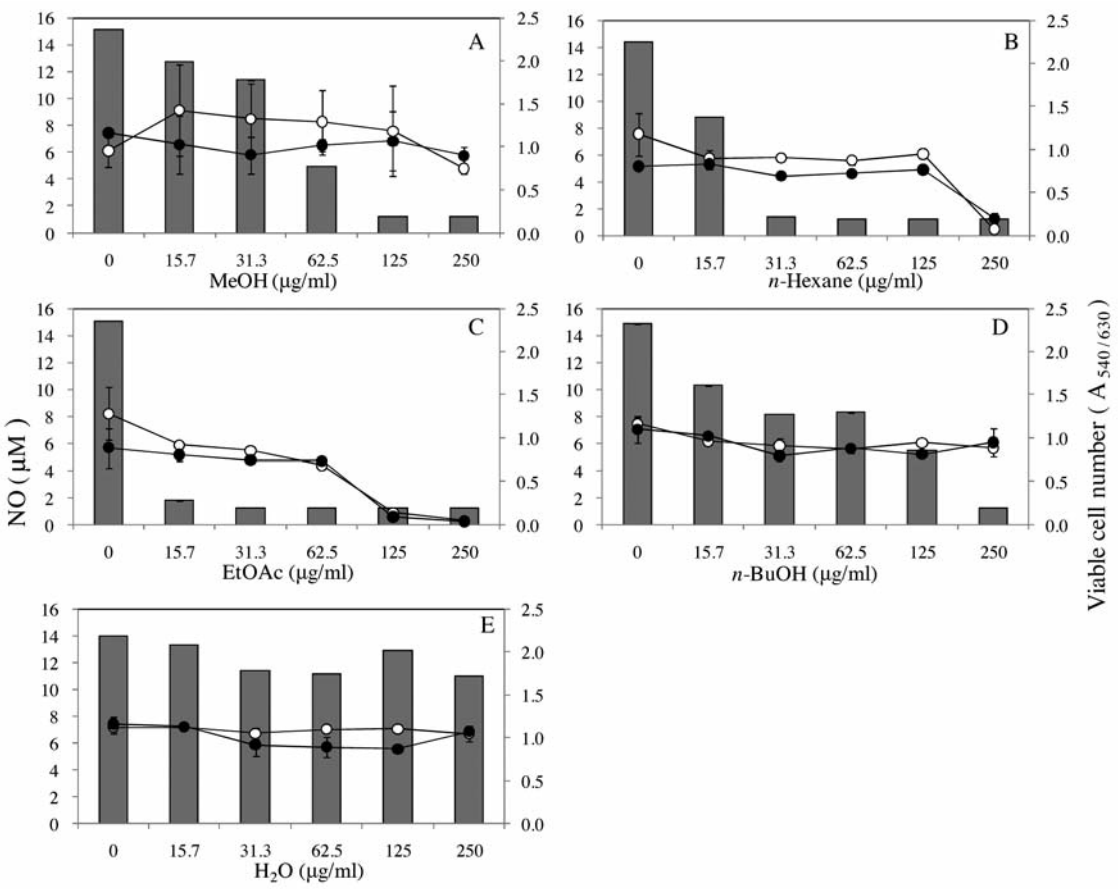

Figure 4. *Effect of MeOH extracts of O. Marie Noel 'Velano' on NO production in RAW264.7 cells. Cells were incubated for 24 hours with MeOH (A) extract of the bulb of O. Marie Noel 'Velano', or n-hexane (B), EtOAc (C), n-BuOH (D) or H_2O (E) fraction of the MeOH extract in the presence or absence of LPS (100 ng/ml). The viable cell number (●: +LPS; ○: LPS) and extracellular NO concentration (bar) were then determined by MTT and Griess methods, respectively. Only NO production data in the presence of LPS are shown, since none of the extracts induced detectable NO production. Mean±S.D. of triplicate assays.*

Table I. *Cytotoxic activity of O. Marie Noel 'Velano' extracts against cultured human tumor and normal cells.*

	CC_{50} (μg/ml)							
	Tumor cell lines				Normal cells			
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	TS
MeOH extract	>246±7	>221±32	188±9	106±29	>243±13	>239±19	>243±12	1.3
n-Hexane fraction	190±12	171±35	176±36	56.0±4.0	>221±51	>230±29	>230±34	1.5
EtOAc fraction	106±39	95.1±39.9	45.1±8.9	35.0±20.8	142±84	133±80	166±59	2.1
n-BuOH fractiont	>250	>250	>250	>250	>250	>250	>250	1.0
H_2O layer	>250	>250	>250	>249±1	>250	>250	>250	1.0

Cells were incubated for 48 hours with various concentrations of test samples. CC_{50}, 50% cytotoxic concentration. TS, tumor specificity. Mean±S.D. of three independent experiments.

fractions (TS=1) (Table I). It should be noted that the order of sensitivity to the EtOAc fraction was in the same order as that to the MeOH extract: HL-60 (CC_{50}=35 μM) > HSC-4 (CC_{50}=45 μM) > HSC-3 (CC_{50}=95 μM) > HSC-2 (CC_{50}=106 μM) > normal cells (HGF, HPC, HPLF) (CC_{50}=133-166 μM) (Table I).

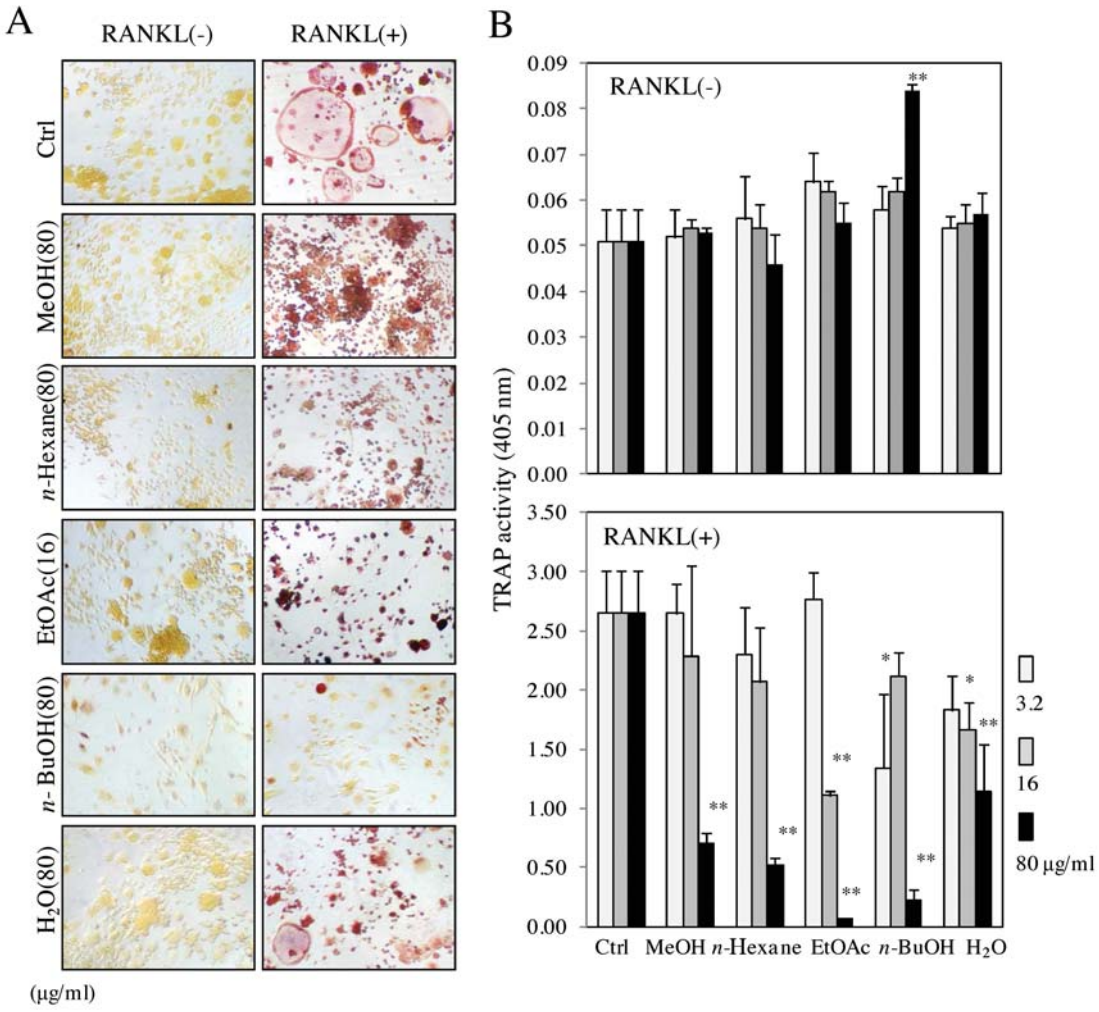

Figure 5. *Effects of MeOH extracts of O. Marie Noel 'Velano' on osteoclast differentiation. RAW264.7 cells were cultured for 4 days in the presence or absence of RANKL with MeOH extract, or n-hexane, EtOAc, n-BuOH fraction or H_2O layer. A: Representative morphology of the TRAP-stained cells. B: TRAP activity on the medium. All samples contained 0.2% DMSO as a vehicle control. Mean±SD of quadruplicated assays. *p<0.05, **p<0.01, compared to control.*

UV protection activity. One minute exposure of the HSC-2 cells to UV irradiation induced irreversible cell death after 48 hours incubation (Figure 3). Addition of the EtOAc fraction to the irradiation buffer markedly protected the cells from the cytotoxicity induced by UV irradiation. The UV protection effect of the EtOAc fraction was increased with increasing concentrations up to 500 μg/ml, where some cytotoxicity was observed (Figure 3C). The *n*-BuOH fraction showed slightly lower UV protective activity (Figure 3D). The MeOH extract, *n*-hexane and H_2O fractions (Figure 3A, B and E, respectively) displayed no UV-protective effects.

NO production by macrophage-like cells. Treatment of the RAW264.7 cells with LPS significantly elevated the extracellular concentration of NO from the background level to 14-15 μM (Figure 4). Simultaneous addition of the EtOAc fraction (Figure 4C) inhibited the LPS-stimulated NO production (EC_{50}=8.9 μg/ml, CC_{50}=90.6 μg/ml, SI=10.2). The *n*-hexane fraction (Figure 4B), which was much less cytotoxic (CC_{50}=205.2 μg/ml), produced a comparable SI value (SI=10.7). On the other hand, crude MeOH extract (SI=5.0, Figure 4A), *n*-BuOH (SI=3.1, Figure 4D) and H_2O (SI=1.0, Figure 4E) fractions showed much less or no inhibitory activity on the NO production (Table II).

Effect on osteoclastogenesis. Treatment of the RAW264.7 cells with RANKL stimulated the production of osteoclasts (Figure 5A), whereas the MeOH extract and its fractions except for the *n*-BuOH fraction were inactive in osteoclastogenesis (Figure 5B, upper panel). On the other

Table II. *Effect of O. Marie Noel 'Velano' extracts on NO production in LPS-activated RAW264.7 cells.*

	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
MeOH extract	>250	49.9	>5.0
n-Hexane fraction	205	19.1	10.7
EtOAc fraction	90.6	>8.9	>10.2
n-BuOH fraction	>250	81.4	>3.1
H_2O layer	>250	>250	>1.0

Cells were incubated for 24 hours with various concentrations of test samples. CC_{50}, 50% cytotoxic concentration. EC_{50}, 50% effective concentration. SI, selectivity index.

hand, the MeOH extract and its fractions significantly ($p<0.01$) inhibited the RANKL-stimulated osteoclastogenesis at higher concentrations (16 or 80 μg/ml) (Figure 5B, lower panel). Among them, the EtOAc fraction showed the most potent inhibitory effect, followed by the *n*-BuOH fraction > *n*-hexane fraction > MeOH extract >> H_2O fraction.

Discussion

The present study demonstrated, for the first time, several biological activities of the MeOH extracts of the bulb of *O.* Marie Noel 'Velano'. Among the four different fractions of the MeOH extract, the EtOAc fraction showed several interesting biological activities: higher cytotoxicity against human tumor cells (three oral squamous cell carcinomas and one promyelocytic leukemia) than against human normal oral cells, with a TS value of 2.1 (Table I); protection of the cells from UV-induced damage (Figure 3); inhibition of NO production by LPS-activated RAW264.7 cells (Figure 4, Table II) and RANKL-stimulated osteoclastogenesis (Figure 5), suggesting its possible anti-inflammatory activity. However, the EtOAc fraction was found to be the most cytotoxic (Table I). We have recently separated the EtOAc fraction by silica gel column chromatography into a flow through fraction and adsorbed fractions which were then eluted stepwise, in order to separate the active principle(s) from the cytotoxic substance(s). The biological activities of these fractions will be reported elsewhere.

The present study also demonstrated that the *n*-BuOH fraction also showed minor stimulation of osteoclastogenesis although this fraction inhibited the RANKL-induced osteoclastogenesis, suggesting that the *n*-BuOH fraction contains both stimulator and inhibitor of osteoclastogenesis. Further fractionation is necessary to separate these substances.

Acknowledgements

We are grateful to Nichirei Garden Corporation, Japan, for their kind supply of *O.* Marie Noel 'Velano'.

References

1 Gutierrez RMP, Gonzalez AMN, Baez EG and Diaz SL: Studies on the constituents of bulbs of the orchid *Prosthechea michuacana* and antioxidant activity. Chem Nat Comp *46*: 554-561, 2010.
2 Yang H, Sung SH and Kim YC: Antifibrotic phenanthrene of *Dendrobium nobile* stems. J Nat Prod *70*: 1925-1929, 2007.
3 Kovács A, Vasas A and Hohmann J: Natural phenanthrenes and their biological activity. Phytochemistry *69*: 1084-1110, 2008.
4 Takagi S, Yamaki M and Inoue K: Antimicrobial agents from *Bletilla striata*. Phytochemistry *22*: 1011-1015, 1983.
5 Fan C, Wang W, Wang Y, Qin G and Zhao W: Chemical constituents from *Dendrobium densiflorum*. Phytochemistry *57*: 1255-1258, 2001.
6 Zhao W, Ye Q, Tan X, Jiang H, Li X, Chen K and Kinghorn AD: Three new sesquiterpene glycosides from *Dendrobium nobile* with immunomodulatory activity. J Nat Prod *64*: 1196-1200, 2001.
7 Ikeda Y, Nonaka H, Furumai T and Igarashi Y: Cremastrine, a pyrrolizidine alkaloid from *Cremastra appendiculata*. J Nat Prod *68*: 572-573, 2005.
8 Kantoh K, Ono M, Nakamura Y, Nakamura Y, Hashimoto K, Sakagami H and Wakabayashi H: Hormetic and anti-radiation effects of tropolone-related compounds. In Vivo *24*: 843-851, 2010.
9 Ralph P and Nakoinz I: Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. J Immunol *119*: 950-954, 1977.
10 Zhou L, Hashimoto K, Satoh K, Yokote Y, Kitajima M, Oizumi T, Oizumi H and Sakagami H: Effect of *Sasa senanensis* Rehder extract on NO and PGE_2 production by activated mouse macrophage-like RAW264.7 cells. In Vivo *23*: 773-777, 2009.
11 Kawano M, Sakagami H, Satoh K, Shioda S, Kanamoto T, Terakubo S, Nakashima H and Makino T: Lignin-like activity of *Lentinus edodes* mycelia extract (LEM). In Vivo *24*: 543-551, 2010.

Received November 29, 2010
Revised February 2, 2011
Accepted February 3, 2011