**in vivo** *26*: 993-1000 (2012)

# In Search of New Biological Activities of Isolates from *Odontoglossum* Harvengtense 'Tutu'

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**Abstract.** *Background: In the current study, we isolated four known compounds, two phenanthrenes, 2,5-dihydroxy-4,9-dimethoxy phenanthrene [**1**] and 4-methoxyphenanthrene-2,7-diol (flavanthrinin) [**2**], one phenanthrenequinone, 5-hydroxy-2,3-dimethoxy-1,4-phenanthrenequinone [**3**], and one flavone, 3,5,7-trihydroxyflavone (galangin) [**4**], from the ethyl acetate (EtOAc) extract of Odontoglossum Harvengtense 'Tutu' through bioassay-guided fractionation, and investigated their biological activities. Materials and Methods: The isolated compounds were identified with spectroscopic analysis and through comparison to literature values. Cytotoxic activity towards human tumor and normal cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Nitric oxide (NO) was determined by the Griess method. Radical scavenging activity was determined by electron spin resonance (ESR) spectroscopy. Osteoclastogenesis was monitored by tartrate-resistant acid phosphatase (TRAP) activity. Results: The compounds had slightly higher cytotoxicity towards human oral squamous cell carcinoma and leukemia cell lines as compared with human normal oral cells, yielding a tumor specificity value of 1.1-2.7. Among these four compounds, **1** most potently inhibited the lipopolysaccharide (LPS)-stimulated NO production and the receptor activator of nuclear factor-κB ligand (RANKL)-stimulated osteoclastogenesis by mouse macrophage-like RAW264.7 cells. Micromolar concentrations of **1** scavenged the NO radical produced from 1-hydroxy-2-oxo-3-(N-3-methyl-3-aminopropyl)-3-methyl-1-triazene. Conclusion: The present study demonstrated, for the first time, that **1** inhibited both macrophage activation and osteoclast differentiation, suggesting its possible anti-inflammatory action.*

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*Key Words: Odontoglossum* Harvengtense 'Tutu', cytotoxicity, activated macrophages, osteoclastogenesis.

The Orchidaceae family is widely considered as the largest plant family, with over 800 genera and at least 24,000 species (1). Various pharmacologically active components have been isolated from some orchid species. During our cytotoxicity screening of the extracts from some orchid plants, we found that an EtOAc-soluble fraction of the methanolic extract of *Odontoglossum* Harvengtense 'Tutu' demonstrated significant cytotoxicity towards some human oral cavity cancer cell lines, as compared with human normal oral cells (2). We have isolated four known compounds, two phenanthrenes [**1** and **2**], one phenanthrenequinone [**3**] and one flavone [**4**] from the dried bulb of *O.* Harvengtense 'Tutu' (Figure 1). By spectroscopical analyses, we identified these compounds as 2,5-dihydroxy-4,9-dimethoxy phenanthrene [**1**] (3), 4-methoxyphenanthrene-2,7-diol (flavanthrinin) [**2**] (3), one phenanthrenequinone, 5-hydroxy-2,3-dimethoxy-1,4-phe-nanthrenequinone [**3**] (4), and one flavone, 3,5,7-trihydroxyflavone (galangin) [**4**] (5, 6) (Figure 1). These compounds showed several biological activities, such as antioxidant (3), anti-inflammatory (7), antiviral and antitumor activities (8). To search for new biological activities, in the current stuy, we investigated their effects on the growth of normal oral (human gingival fibroblast, HGF; pulp cells, HPC; periodontal ligament fibroblast, HPLF) and tumor cells (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4, Ca9-22, human promyelocytic leukemia HL-60), and the activation of mouse macrophage-like cells RAW264.7, upon stimulation with lipopolysaccharide (LPS), and the inhibition of osteoclastogenesis induced by receptor activator of nuclear factor-κB ligand (RANKL).

## Materials and Methods

*Materials.* The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA); fetal bovine serum

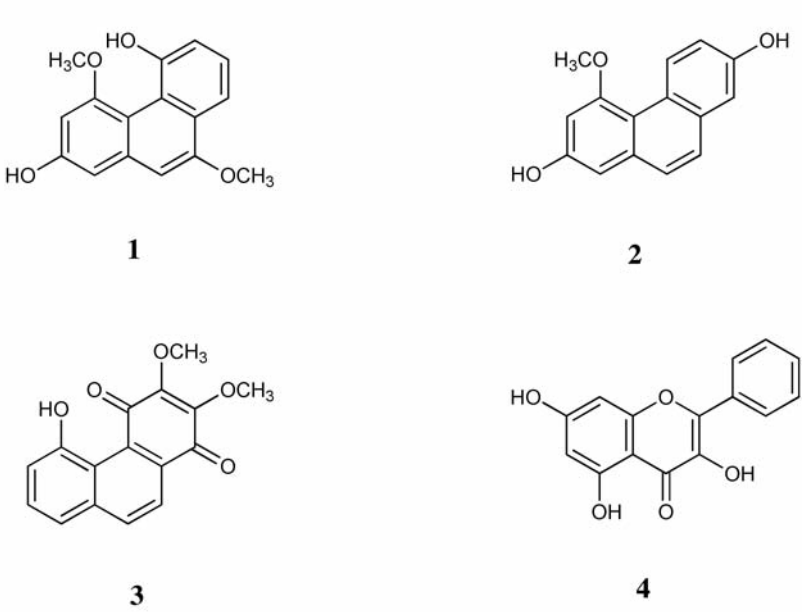


Figure 1. *Structures of compounds **1-4** isolated from Odontoglossum Harvengtense 'Tutu'.*

(FBS, JRH Bioscience, Lenexa, KS, USA); LPS from *Escherichia coli* (serotype 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD) (Sigma Chem Co., St Louis, MO, USA); 5-fluorouracil (5-FU) (Kyowa, Tokyo, Japan); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Inc., Osaka, Japan); RANKL (R&D Systems, Minneapolis, MN, USA); (4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy1-3-oxide (carboxy-PTIO; a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene [NOC-7; a nitric oxide (NO) generator], diethylenetriaminepenta-acetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (Dojin, Kumamoto, Japan).

Bulbs of *O*. Harvengtense 'Tutu' (Orchidaceae) were supplied from Orchid Garden Co., Ltd., Nagano Prefecture, Japan, during April of 2009. Specimen were proven and identified by Mr. H. Sumiyoshi, Orchid Garden Co., Ltd. and a voucher specimen (#20090618) was also deposited at the Medicinal Plant Garden of Josai University.

*General experimental procedures*. One- and two-dimensional nuclear magnetic resonance (NMR) spectra were recorded on an Agilent 400-MR NMR spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA), at room temperature using standard pulse sequences. Electro ionization-mass spectra (EI-MS) were measured with a JEOL JMN 700 spectrometer (JEOL Ltd., Tachikawa, Tokyo, Japan). Open column chromatography was performed on Silica gel 60 (Merck KGaA, Darmstadt, Germany). High-performance liquid chromatography (HPLC) was performed with Senshu Pak octadecyl silyl (ODS) silica gel column (4.6 mm i.d. ×150 mm for analysis, 10 mm i.d. ×250 mm for preparation; Senshu Scientific co., ltd., Suginami-ku, Tokyo, Japan).

*Extraction and isolation*. The air-dried bulbs (956 g) of *O*. Harvengtense 'Tutu' were extracted with MeOH three times under reflux for 3 h. The methanolic extract (106 g) was fractionated by sequential organic solvent extraction, as described previously (2). The EtOAc-soluble fraction of the methanolic extract (8.73 g) was subjected to silica gel chromatography, eluted with a $CHCl_3$-MeOH mixture (20:1→10:1→5:1→1:1) to give 15 fractions (Fr. A–O). Fr. E (943.1 mg) was chromatographed on a silica gel column by gradient elution with *n*-hexane-diethyl ether-MeOH mixture (7:3:0→1:1:0 →0:1:0→0:1:1) to obtain 12 fractions (Fr. E-a–E-l). Fr. E-c (31.3 mg) was purified with HPLC on an ODS column using acetonitrile-water solvent system to gain 3,5,7-trihydroxyflavone (galangin) (**4**; 1.7 mg) (Figure 1). Fr. F (923.2 mg) was re-chromatographed on a silica gel column, eluting with *n*-hexane-diethyl ether-MeOH mixture (7:3:0→1:1:0→0:1:0→0:1:1) to give 10 fractions (Fr. F-a–F-j). Further purifications of Fr. F-b (60.5 mg), Fr. F- f (28.3 mg) and Fr. F-g (91.0 mg) by reversed-phase HPLC with acetonitrile–water mixture as eluent resulted in the purification of compounds 2,5-dihydroxy-4,9-dimethoxy phenanthrene (**1**; 4.5 mg), 4-methoxyphenanthrene-2,7-diol (flavanthrinin) (**2;** 9.5 mg) and 5-hydroxy-2,3-dimethoxy-1,4-phenanthrenequinone (**3**; 14.1 mg), respectively.

*Cell culture*. Human promyelocytic leukemia HL-60 cells were provided by Prof. K. Nakaya, Showa University, Japan. Human oral squamous cell carcinoma cell lines (HSC-2, HSC-3, HSC-4, Ca9-22) were provided by Prof. M. Nagumo, Showa University. Normal human oral cells, HGF, HPC and 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 (9). Since normal oral cells have a limited lifespan of 43-47 population doubling levels (PDL) (9), they were used at 8-15 PDL. Mouse macrophage-like RAW264.7 cells (10) were purchased from Dainippon Sumitomo Pharma, Osaka, Japan. 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 atmosphere with 5% $CO_2$. 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 by 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 five tumor cell lines were similarly trypsinized and subcultured.

*Assay for cytotoxic activity*. The cells ($3\times10^3$ cells/well, 0.1 ml/well) were seeded in 96-microwell plates (Becton Dickinson and Co., Franklin Lakes, NJ, USA) and incubated for 48 h to allow for cell attachment. Near-confluent cells were treated for 48 h with different 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 (2). In brief, control and sample-treated cells were incubated for 4 h 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).

*Macrophage activation*. RAW264.7 cells were cultured as an adherent monolayer culture in DMEM supplemented with 10% heat-inactivated FBS under a humidified atmosphere with 5% $CO_2$. When the cells had grown to confluency, they were detached by being splashed with the medium through the pipette, and seeded at a lower cell density. We did not collect the cells by scraping them out from the plate with a rubber policeman, since the scraping procedure damaged the cells (unpublished data).

RAW264.7 cells were inoculated at $2\times10^6$/ml (100 μl) in a 96-microwell plate (Becton Dickinson and Co.) and incubated for 2 h. Near-adherent cells were replenished with fresh culture medium and then treated for 24 h with the indicated concentrations of test samples in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 μg/ml). The viable cell number was then determined by the MTT method, as described above, and the concentration of NO released into the culture medium was quantified by the Greiss reagent, using the standard curve of $NO_2^-$. The $CC_{50}$ and the concentration that inhibited the LPS-stimulated NO production by 50% (50% inhibitory concentration: $EC_{50}$) were then determined from the dose–response curve. The efficacy of inhibition of NO production was evaluated by the selectivity index (SI), which was calculated using the following equation: $SI=CC_{50}/IC_{50}$.

*Radical-scavenging activity*. The radical intensity of samples was determined at 25˚C, using electron spin resonance (ESR) spectroscopy (X-band, 100 kHz modulation frequency, JEOL JES REIX, JEOL Ltd., Tachikawa, Tokyo, Japan).

The superoxide anion (in the form of DMPO-OOH), produced by the HX-XOD reaction (total volume: 200 μl) [2 mM HX in 50 μl of 0.1 M phosphate buffer (PB) (pH 7.4), 20 μl of 0.5 mM DETAPAC, 30 μl of 10% DMPO, 40 μl of sample, 30 μl of PB, 30 μl of XOD (0.5 U/ml in PB)] was determined (microwave power: 16 mW, gain: 630) (11).

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 60 μM NOC-7, was determined in 0.1 M PB (pH 7.4) in the presence of 30% DMSO (microwave power and gain: 5 mW and 400, respectively). When NOC-7 and carboxy-PTIO were mixed, NO was oxidized to $NO_2$ and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. Samples were added 3 min after mixing. The NO radical intensity was defined as the ratio of the signal intensity of the second peak of carboxy-PTI to that of MnO, and expressed as the ratio to the height of MnO, an external marker (12).

*Assay for osteoclast differentiation*. RAW264.7 cells ($5\times10^4$ cells/ml) were cultured in a 96-well plate (0.1 ml/well) with α-minimum essential medium (α-MEM) (Wako Pure Chemicals, Osaka, Japan) containing 10% FBS in the presence or absence of receptor activator of NF-κB ligand (RANKL; 10 ng/ml; R&D Systems) with or without test compounds. After three days in culture, measurement of tartrate-resistant acid phosphatase (TRAP) activity of the medium and TRAP staining were performed as described previously (2, 13).

*Measurement of TRAP activity and TRAP-staining*. TRAP activities of osteoclasts were 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%), EDTA (6 mM), and NaCl (600 mM). The reaction was terminated by the addition of 30 μl of NaOH (300 mM) and the absorbance was then measured at 405 nm. TRAP histochemical staining of the cells was performed using a leukocyte acid phosphatase kit (Sigma-Aldrich). Cultured cells were fixed with 100% methanol for 1 min at room temperature and air-dried. After TRAP staining, TRAP-positive multinucleated (more than three nuclei) cells were photographed under phase-contrast microscopy (2, 13).

*Statistical treatment*. Experimental values are expressed as the mean±standard deviation (SD). Statistical analysis was performed by using Student's *t*-test. A *p*-value $<0.05$ was considered to be significant.

## Results

*Identification of isolates **1**-**4***. The **1**, **2**, **3** and **4** (Figure 1) were identified on the basis of spectroscopic analyses including NMR, MS and through comparison with literature values.

*Cytotoxicity*. The compounds exhibited slightly higher cytotoxicity towards human oral squamous cell carcinoma and leukemia cell lines, as compared with human oral normal cells, yielding a tumor specificity of 1.1-2.7 (Table I). Compound **1** exhibited the highest tumor specificity, followed by **4>2>3**.

*Inhibition of NO production*. Among these four compounds, **1** most potently inhibited LPS-stimulated NO production by mouse macrophage-like RAW264.7 cells (SI=9.5), followed by **2**, **3** and **4** (Figure 2, Table II). The reduced amount of NO in the culture medium was due either to reduced synthesis or enhanced degradation or scavenging.

Table I. *Cytotoxicity of compounds **1**-**4**. The cells were incubated for 48 h with or without different concentrations of test samples, and the 50% cytotoxic concentration ($CC_{50}$) value was determined by the MTT method. Each value represents the mean of triplicate assays. SD <10%. 5-FU: 5-Fluorouracil, PDL: population doubling level.*

| | $CC_{50}$ (μM) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tumor cell lines | | | | | | Normal cells | | | | | | |
| Compound | HSC-2 | HSC-3 | HSC-4 | Ca 9-22 | HL-60 | Mean (A) | HGF 13PDL | HGF 28PDL | HPC 30PDL | HPLF 11PDL | HPLF 31PDL | Mean (B) | TS (B/A) |
| **1** | 75.6 | 112.6 | 83.4 | 25.3 | 22.6 | 63.9 | 220.3 | 182.7 | 121.5 | 169.9 | 160.6 | 171.0 | 2.7 |
| **2** | 105.0 | 96.4 | 101.3 | 110.7 | 49.2 | 92.5 | 151.2 | 168.6 | 122.7 | 146.9 | 139.3 | 159.9 | 1.7 |
| **3** | 32.5 | 78.0 | 52.9 | 38.5 | 20.0 | 44.4 | 56.0 | 26.2 | 65.8 | 45.9 | 40.5 | 46.9 | 1.1 |
| **4** | 65.6 | 120.0 | 78.3 | 92.9 | 78.6 | 87.1 | 287.2 | <201.3 | 184.2 | 291.2 | <73.4 | <207.5 | <2.4 |
| 5-FU | 136.9 | 68.2 | 68.2 | <12.5 | 72.2 | 86.4 | >400 | >400 | >400 | >400 | >400 | >400 | >4.4 |

ESR spectroscopy showed that **2** most effectively scavenged the NO radical, produced from NOC-7 (NO generator) ($IC_{50}$=2 μM), followed by **1** ($IC_{50}$=19 μM) and **3** ($IC_{50}$=350 μM) (Table III). The NO radical-scavenging activity of **4** could not be determined due to non-specific binding with assay reagents for NO radical detection. We found that all four compounds poorly scavenged superoxide anion ($IC_{50}$=250-1300 μM), which is known to interact with NO (14). It should be noted that **1** and **2**, which effectively scavenged NO, poorly scavenged superoxide anion, whereas **3**, which poorly scavenged NO, most effectively scavenged superoxide anion (Table III), confirming the existence of an interaction between NO and superoxide anion. These results suggest that the inhibition by **1** of NO production, by LPS-activated macrophages, is mostly due to its NO radical-scavenging activity.

*Osteoclastogenesis*. The mouse macrophage-like cell line RAW 264.7 has been shown to readily differentiate into osteoclasts upon exposure to RANKL. We previously showed that crude extracts from the bulb of *O*. Harvegtense 'Tutu' have an inhibitory activity on osteoclast differentiation in RAW264.7 cells (2). To determine which compound isolated from 'Tutu' inhibits osteoclastogenesis, we evaluated the effects of compounds on the osteoclast formation the investigated of RAW264.7 cells. As shown in Figure 3A, TRAP activity of the medium of the RANKL-stimulated cells (R) was increased as compared with the RANKL-untreated control (N). The TRAP activities induced by RANKL were inhibited in a dose-dependent manner by **1** and to a lesser extent by **2**. On the other hand, **3** and **4** did not inhibit RANKL-induced differentiation. None of the compounds, including **1**, altered the TRAP activity of cells not stimulated by RANKL. As shown in Figure 3B, TRAP-positive multinucleated cells were observed in the culture of cells with RANKL treatment, while treatment with **1** inhibited the production of RANKL-stimulated multinucleated osteoclasts, suggesting that **1** is a major determinant inhibiting osteoclast formation.

Table II. *Inhibition of NO production in lipopolysaccharide (LPS)-activated RAW264.7 cells by compounds **1**-**4**. Three independent experiments (Exp. I, II and III) were performed, that gave reproducible results. The 50% cytotoxic concentration ($CC_{50}$) and 50% inhibitory concentration ($EC_{50}$) values were determined from the dose-response curve (such as shown in Figure 2). SI:Selectivity index.*

| Compound | | $CC_{50}$ (μM) | $EC_{50}$ (μM) | SI |
|---|---|---|---|---|
| **1** | Exp.1 | 159.5 | 11 | 14.5 |
| | Exp.2 | 148.6 | 19.6 | 7.6 |
| | Exp.3 | 267.5 | 41.8 | 6.4 |
| | Mean±SD | 191.9±65.7 | 24.1±15.9 | 9.5±4.4 |
| **2** | Exp.1 | 208.1 | 49.8 | 4.2 |
| | Exp.2 | 166 | 39.1 | 4.2 |
| | Exp.3 | 216.5 | 73.6 | 2.9 |
| | Mean±SD | 196.7±27.1 | 54.2±17.7 | 3.8±0.8 |
| **3** | Exp.1 | 386.6 | 92.2 | 4.2 |
| | Exp.2 | 268.1 | 82.1 | 3.3 |
| | Exp.3 | >400 | 145.5 | >2.7 |
| | Mean±SD | >327.4±83.8 | 106.6±34.1 | >3.4±0.8 |
| **4** | Exp.1 | 176.1 | 210.1 | 0.8 |
| | Exp.2 | 172.8 | 85.7 | 2 |
| | Exp.3 | 224.2 | 104.8 | 2.1 |
| | Mean±SD | 191.0±28.8 | 133.5±67.0 | 1.6±0.7 |

## Discussion

The present study demonstrated that all four compounds isolated here, exhibited some tumor-specific cytotoxicity (TS=1.1-2.7), although the TS values were considerably lower than the one of 5-FU (TS>4.4) (Table I), one of the popular antitumor agents against oral squamous cell carcinoma (15, 16). Among these compounds, **1** had the

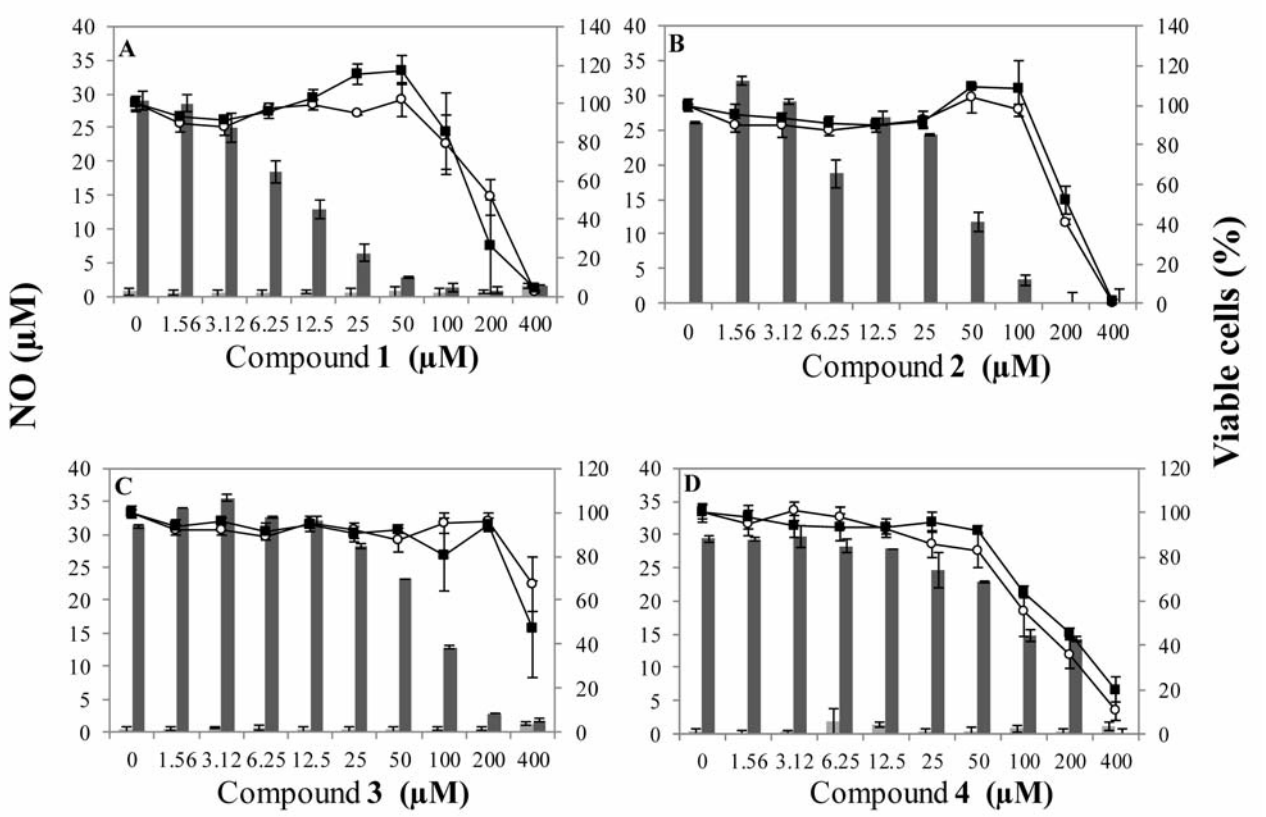


Figure 2. *Inhibition of NO production by compounds **1-4** in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Bars and dots present the NO production and viable cell numbers, respectively, in the presence (dark color) or absence (light or white color) of LPS. Two repeated experiments performed independently (Exps. II and III) gave similar results (not shown). The 50% cytotoxic concentration ($CC_{50}$) and 50% inhibitory concentration ($EC_{50}$) values are listed in Table II.*

highest tumor specificity, followed by **4>2>3**. It remains to be investigated what type of cell death was induced by **1**.

We found that **1** inhibited NO production by LPS-stimulated macrophages to the greatest extent. This finding was reproducible in three independent experiments (Table II). This compound was found to scavenge the NO radical (produced from NOC-7) ($EC_{50}$=19 μM) at a concentration slightly lower than the one needed to inhibit NO production stimulated by LPS ($EC_{50}$=24 μM), indicating that the inhibition of NO production is mostly due to the scavenging of the NO released from the activated macrophages. However, the possibility of the inhibition of inducible NO synthase (iNOS) protein expression by **1** has not yet been excluded.

We also found that **1** inhibited the RANKL-stimulated osteoclastogenesis most efficiently among the four compounds tested. As far as we know, the report of biological activity of **1** has been limited to just one article that investigated its antioxidant activity (3). The present study demonstrated, for the first time, that **1** inhibited both macrophage activation and differentiation towards maturing osteoclasts, suggesting its possible anti-inflammatory action.

Table III. *NO radical-scavenging activity of compounds **1-4**. Each value represents the mean±S.D. of triplicate assays. N.D.: Not determined.*

| | Radical-scavenging activity towards | |
|---|---|---|
| Compound | NO ($IC_{50}$, μM ) | $O_2^-$ ($IC_{50}$, μM) |
| **1** | 19±0.8 | 1300±85 |
| **2** | 2±0.14 | 1200±44 |
| **3** | 350±9.1 | <250 |
| **4** | N.D. | 602±40 |

**3** was recently isolated as a new compound and its structure was determined by our group (4). The present study also demonstrated that **3** ($IC_{50}$<250 μM) had more than 5-times higher superoxide-scavenging activity than **1** ($IC_{50}$=1300 μM) (Table III). The application of **3** as new antioxidant may be promising.

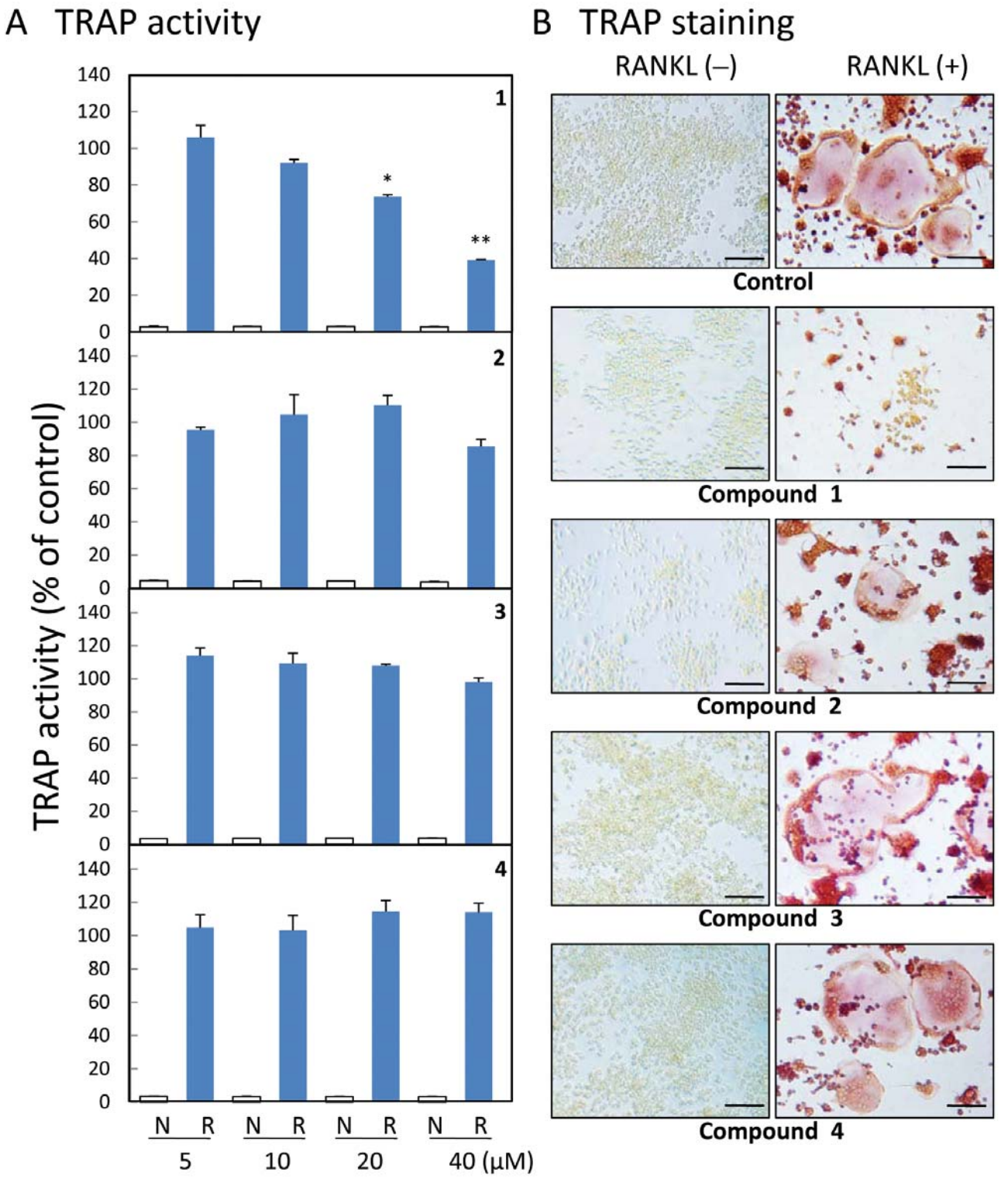


Figure 3. *Effect of compounds **1-4** on osteoclastogenesis of RAW264.7 cells. After culture in the presence or absence of the receptor activator of nuclear factor-κB ligand (RANKL) with or without the investigated compounds, tartrate-resistant acid phosphatase (TRAP) activity of the medium (A) and TRAP staining (B) were investigated. N: Untreated control; R: RANKL treated. *p<0.05, **p<0.01. Bars, 100 μm.*

## Acknowledgements

Thanks are due to Orchid Garden Co., Ltd. Japan for their kind supply of *Odontoglossum* Harvengtense 'Tutu'.

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*Received June 13, 2012*
*Revised September 14, 2012*
*Accepted September 17, 2012*