New Biological Activities of Rhinacanthins from the Root of *Rhinacanthus nasutus*

HARUKA HORII[1], RYUICHIRO SUZUKI[1], HIROSHI SAKAGAMI[2], MINEKO TOMOMURA[3], AKITO TOMOMURA[3] and YOSHIAKI SHIRATAKI[1]

[1]Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan; Divisions of [2]Pharmacology and [3]Biochemistry, Meikai University School of Dentistry, Sakado, Saitama, Japan

Abstract. *Background: We recently reported that the ethyl acetate (EtOAc)-soluble fraction of the methanol extract of the root of Rhinacanthus nasutus showed tumor-specific non-apoptotic cytotoxicity and antiosteoclastogenic activity. In the present study, we investigated whether five rhinacanthins, mostly isolated from the EtOAc-soluble fraction of this plant, are responsible for these activities. Materials and Methods: The cytotoxic activity was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. The 50% cytotoxic concentration (CC_{50}) was determined by the dose-response curve. Tumor specificity (TS) was determined by the ratio of the mean CC_{50} for normal cells to that of tumor cell lines. DNA fragmentation was assayed by agarose gel electrophoresis. Caspase-3 activation was monitored by substrate cleavage assay. Osteoclastogenesis was monitored by tartrate-resistant acid phosphatase (TRAP) activity in receptor activator of NF-κB ligand (RANKL)-stimulated bone marrow-derived macrophages. Results: Among five rhinacanthins (rhinacanthin C, G, N and Q, and rhinacanthone), rhinacanthin C exhibited the highest tumor specificity (TS=15.2). Rhinacanthin C did not induce internucleosomal DNA fragmentation nor caspase-3 activation, suggesting non-apoptotic cell death. Rhinacanthin C most potently inhibited the RANKL-stimulated osteoclastogenesis. Conclusion: The present study suggests that rhinacanthin C may be responsible for the biological activity of the EtOAc-soluble fraction prepared from the methanolic extract of R. nasutus we previously reported on.*

Correspondence to: Professor 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: Rhinacanthus nasutus, cytotoxicity, apoptosis induction, osteoclastogenesis, HSC-2, HSC-3, HSC-4, HL-60 cells.

The shrub *Rhinacanthus nasutus* (L.) Kurz (Acanthaceae) is widely distributed in Southeast Asian countries and used for the treatment of pneumonia, diabetes, hypertension and skin diseases (1). The root and aerial parts of this plant contains naphthoquinone esters, such as rhinacanthins C, D, N and Q, which exhibit apoptosis-inducing (2-5), antitumor, antiviral (6), antiallergic (7) and anti-inflammatory activities (8, 9). In order to search for new biological activities of the components of this plant, we crudely separated the methanol (MeOH) extract into four fractions [*n*-hexane, ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and aqueous layers] by successive organic solvent extractions and compared their biological activities (10). We found that the EtOAc extract had the highest tumor-specific cytotoxicity, and inhibited the nitric oxide production stimulated by lipopolysaccharide and the osteoclastogenesis stimulated by receptor activator of nuclear factor-κB ligand (RANKL) in mouse macrophage-like RAW264.7 cells (10). When the EtOAc extract was further separated by silica-gel column chromatography into five fractions (Frs.1-5), Fr. 1 eluted with $CHCl_3$-MeOH (50:1) had the highest tumor specificity (TS=3.3) inducing little or no apoptosis in human oral squamous cell carcinoma cell lines (11).

In the present study, we isolated five components (Figure 1), rhinacanthins C [**1**], G [**2**], N [**3**], Q [**4**] and rhinacanthone [**5**] from the MeOH extract of the root of *R. nasutus*, and investigated on their tumor specificity, apoptosis-inducing activity and antiosteoclastogenic activity.

Materials and Methods

Materials. The root of *R. nasutus* was supplied from the Chiayi Grass-Produce Cooperation Farm (Chiayi County, Taiwan, ROC) during the autumn of 2009. This specimen was identified by Professor Y. Shirataki and a voucher specimen (#201006060) was also deposited at the Medicinal Plant Garden of Josai University, Japan.

General experimental procedures. The nuclear magnetic resonance (NMR) spectra were measured on JEOL EX270 spectrometer (JEOL Ltd., Tachikawa, Tokyo, Japan) at room temperature using

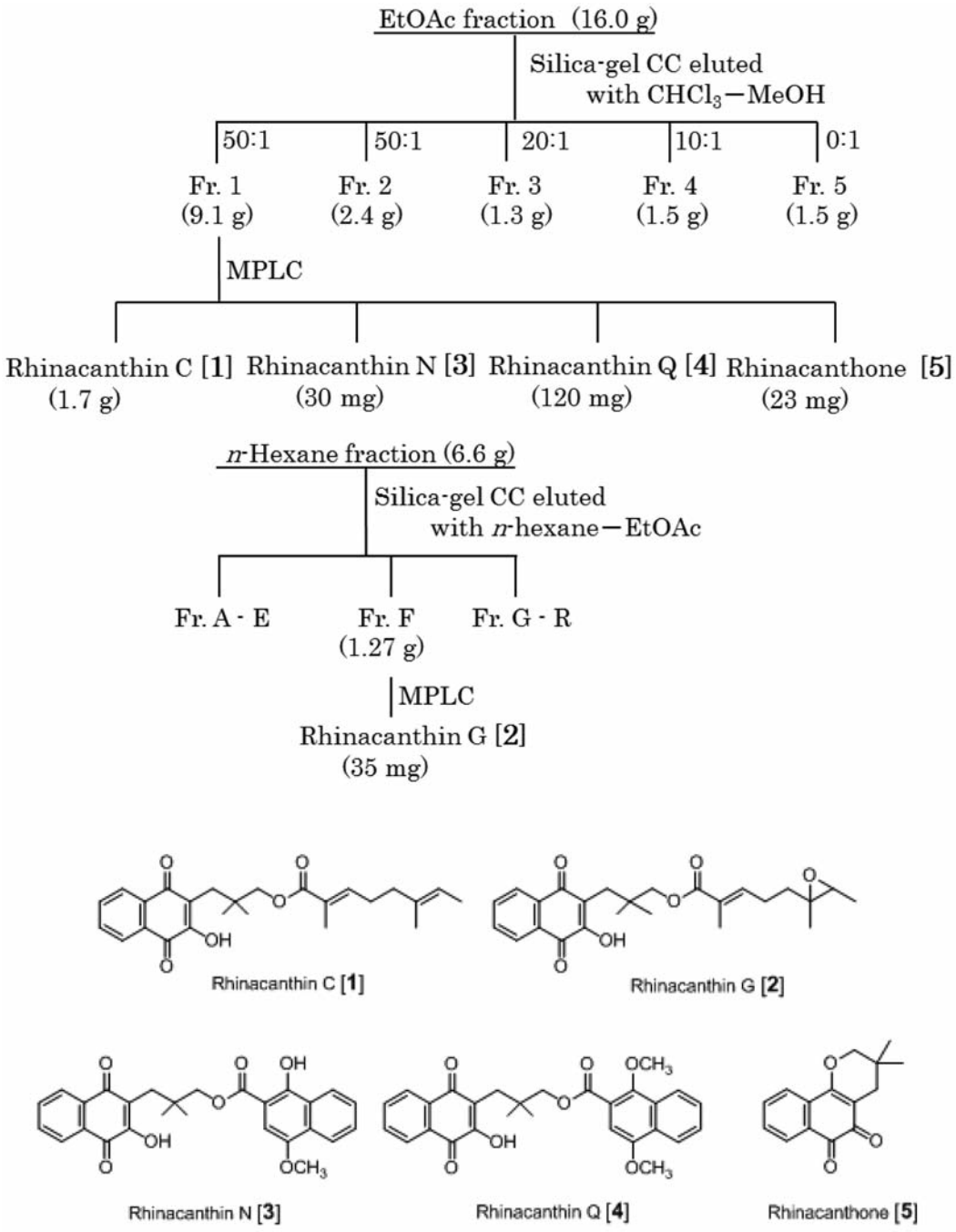

Figure 1. *Flow chart of isolation of five rhinacanthins from the n-hexane and EtOAc fractions of the MeOH extract of root of Rhinacanthus nasutus, and structures of isolated compounds. CC, Column chromatography; MPLC, Medium-pressure liquid chromatography.*

standard pulse sequences. Electron ionization-mass spectra (EI-MS) were measured with a JEOL JMN 700 spectrometer (JEOL Ltd.). Open-column chromatography was performed on Silica gel 60 (Merck KGaA, Darmstadt, Germany). Medium-pressure liquid chromatography (MPLC) purifications were performed on a YAMAZEN YFLC-AI-580 instrument (YAMAZEN Co., Yodogawa-ku, Osaka, Japan).

Extraction and isolation. As shown in Figure 1, the root (1.2 kg) of *R. nasutus* was extracted with MeOH three times under reflux for 3 h. The MeOH extract (83 g) was fractionated by sequential organic solvent extraction, as described previously (10, 11). The EtOAc-soluble portion (16.0 g) was chromatographed on a silica gel column by gradient elution with a $CHCl_3$–MeOH mixture ($CHCl_3$: MeOH=50:1 → 20:1 → 10:1 → 0:1) to give five fractions (Fr. 1-5).

Fr. 1 (9.1 g) was further purified using normal-phase MPLC (silica gel, Hi-Flash 2L, ϕ26×150 mm, *n*-hexane:MeOH=100:0 → 89:11) to give **1** (1.7 g), **3** (30 mg), **4** (120 mg) and **5** (23 mg), respectively. The *n*-hexane-soluble portion (6.6 g) was fractionated with silica gel open-column chromatography eluting with *n*-hexane–EtOAc mixture (*n*-hexane:EtOAc=9:1 → 4:1 → 7:3 → 1:1) to provide eighteen fractions (Fr. A–R). Fr. F (1.27 g) was rechromatographed on MPLC (silica gel, Hi-Flash 2L, ϕ26x150 mm, *n*-hexane:EtOAc=100:0 → 21:4) to yield **2** (35 mg).

Cell culture. Human oral squamous cell carcinoma (OSCC) cell lines (HSC-2, HSC-3, HSC-4) (kindly provided by Prof. Nagumo, Showa University), human promyelocytic leukemia HL-60 cells (kindly provided by Prof. Takeda, Tokyo University of Science), and normal human oral cells [gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF)] (established from first premolar tooth extracted from the lower jaw of a 12-year-old girl) were prepared and cultured, as described previously (10, 11). HL-60 cells were cultured at 37˚C in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under a humidified 5% CO_2 atmosphere. Other cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics.

Assay for cytotoxic activity. The cells (3×10^3 cells/well, 0.1 ml/well) were seeded in 96-microwell plates (Becton Dickenson, 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 extracts in fresh medium. The relative viable cell number of adherent cells (except for HL-60 cells) was then determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method (10, 11). 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 for DNA fragmentation. Cells were lysed with 50 μl of lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 1-2 h at 50˚C, and then mixed with 50 μl NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and 250 μl of ethanol. After centrifugation for 20 min at 15,000 ×*g*, the precipitate was washed with 1 ml of 70% ethanol. After centrifugation for 5 min at 15,000 ×*g*, the precipitate was dried for 10 min, and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 3-5). The isolates were applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The DNA molecular marker (Takara Bio, Otsu, Shiga, Japan) and DNA from apoptotic HL-60 cells induced by ultraviolet (UV) irradiation (6 J/m^2/min), followed by 3 h of incubation, were used for calibration. The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Assay for caspase activation. Cells were washed with phosphate-buffered saline without magnesium and calcium [PBS(−)] and lysed in lysis solution. After standing for 10 min on ice and centrifugation for 5 min at 10,000 ×*g*, the supernatant was collected. The lysate (50 μl, equivalent to 100 μg protein) was mixed with 50 μl reaction buffer (MBL International, Woburn, MA, USA), containing substrates for caspase-3 [DEVD-*p*NA (*p*-nitroanilide)]. After incubation for 3 h at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured with microplate reader.

Assay for osteoclast differentiation. Osteoclasts were differentiated from bone marrow macrophages, as described previously (12). Briefly, bone marrow cells were collected from femur and tibia of ddY mice and cultured with α-minimum essential medium (α-MEM), containing 10% FBS and macrophage-colony stimulating factor (M-CSF, 10 ng/ml; R&D Systems Inc., Minneapolis, MN, USA) on 96-well plates for three days. The cells were further cultured either with M-CSF-alone or M-CSF plus receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL, 10 ng/ml; R&D Systems Inc.), with or without test samples for four days. Measurement of tartrate-resistant acid phosphatase (TRAP) activity of the medium and TRAP staining were performed.

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, St. Louis, MO, USA). 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 (10).

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 less than 0.05 was considered to be significant.

Results

*Identification of isolates **1-5***. We isolated compounds **1**, **3**, **4** and **5** from Fr. 1 of the EtOAc-soluble layer of MeOH extract, and compound **2** from the *n*-hexane-soluble fraction of the methanolic extract of the root of *R. nasutus*. These isolates were identified as rhinacanthin C [**1**] (MW 410.2) (13), G [**2**] (MW 426.2) (14), N [**3**] (MW 460.2) (14) and Q [**4**] (MW 474.2) (5), and rhinacanthone [**5**] (MW 242.1) (15, 16) on the basis of spectroscopic analyses including NMR, MS and comparison with those described in the respective literature (Figure 1).

Cytotoxic activity of rhinacanthins. Among the five rhinacanthins, rhinacanthone showed the highest cytotoxicity against both human tumor and normal cells, yielding the lowest tumor specificity (TS=1.3) (Table I). Rhinacanthin C exhibited higher cytotoxicity against tumor cell lines as compared with normal cells, yielding the highest tumor specificity (TS=15.2). The tumor specificity of rhinacanthin

Table I. *Cytotoxic activity of rhinacanthins from Rhinacanthus nasutus against human normal and tumor cells. Cells were incubated for 48 h with different concentrations of rhinacanthins. CC_{50}, 50% cytotoxic concentration; TS, tumor specificity index. Data are the means±S.D. of three independent experiments. The final concentration of DMSO in the culture medium was less than 1% and did not affect the cellular growth.*

	CC_{50} (μM)							
	Tumor cell lines				Normal cells			
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	TS
Rhinacanthin C	1.66	2.32	12.58	1.15	72.2	53.4	76.3	15.2
Rhinacanthin G	7.16	7.67	12.51	3.38	>73.44	60.3	>73.44	7.9
Rhinacanthin N	3.41	5.37	8.91	2.22	59.8	35.9	>68.02	9.6
Rhinacanthin G	3.99	6.50	12.80	2.59	63.1	50.2	65.6	9.2
Rhinacanthone	3.10	4.92	4.67	5.08	6.24	4.46	6.44	1.3
Peplomycin	2.60	<0.99	7.70	2.76	110.07	2.49	28.89	13.50
Doxorubicin	<0.28	<0.28	<0.28	<0.28	>34.48	1.00	8.62	17.4

G, N and Q were moderate (TS=7.9-9.6). It should be noted that the TS value of rhinacanthin C was comparable with that of peplomycin (TS=13.5) and doxorubicin (TS= >17.4).

Failure of rhinacanthins to induce apoptotic cell death. Rhinacanthin C (1.4 μg/ml, approximately two-fold of CC_{50}) and rhinacanthin N (3.1 μg/ml, approximately two-fold of CC_{50}) did not induce internucleosomal DNA fragmentation, but instead they induced a larger proportion of high molecular weight DNA fragments (indicated by arrows) in HSC-2 cells, regardless of incubation time (6, 24 or 48 h), in contrast to UV-irradiated HL-60 cells, which exhibited a DNA laddering pattern (Figure 2). Treatment of HSC-2 cells with rhinacanthin C did not increase, but rather slightly ($p<0.01$) reduced caspase-3 activity, regardless of incubation time (6, 24 or 48 h), in contrast to UV-irradiation, which significantly activated caspase-3 (Figure 3). These data suggest that rhinacanthin C may have induced non-apoptotic cell death.

Inhibition of osteoclast formation by rhinacanthins. We have reported that the EtOAc fraction of MeOH extract from the root of *R. nasutus* inhibited RANKL-stimulated osteoclast formation in RAW264.7 cells (10). We investigated whether rhinacanthins inhibit the osteoclast formation from bone marrow-derived macrophages. As shown in Figure 4A, rhinacanthin C and G dose-dependently inhibited the RANKL-induced TRAP activity. The inhibitory effect of rhinacanthin C was more potent than that of rhinacanthin G. On the other hand, rhinacanthin N and Q suppressed TRAP activity only at a higher dose. Rhinacanthone had little effect on the RANKL-induced TRAP activity. None of the isolates altered TRAP activity without stimulation with RANKL. As shown in Figure 4B, TRAP-positive multinucleated giant cells were observed in the culture treated with RANKL, while treatment with rhinacanthin C and G inhibited RANKL-induced multinucleated mature osteoclasts. These results suggest that rhinacanthin C and G are major determinants of the inhibition of osteoclastogenesis by *R. nasutus* extract.

Discussion

The present study demonstrated that rhinacanthin C had the highest tumor-specific cytotoxicity (TS=15.2), comparable to that of popular antitumor agents such as peplomycin and doxorubicin (Table I). Purification considerably increased the TS value: 3.1 (EtOAc layer) (10) → 3.3 (Fr. 1) (11) → 15.2 (final step). This suggests that rhinacanthin C is the major compound with tumor-specific cytotoxicity of Fr. 1, EtOAc layer and the MeOH extract of root of *R. nasutus*. We also confirmed that rhinacanthin C did not induce apoptotic cell death, consistent with non-apoptotic cell death induced by Fr. 1 (11) and the MeOH extract (10). The physiological role of larger units of DNA fragments (50-300 kbp) (17) produced by rhinacanthin C in HSC-2 cells is unclear. The failure of apoptosis induction by rhinacanthin C may be due to the presence of the α,β-unsaturated ketone structure in rhinacanthin C, since the α,β-unsaturated ketones such as 1-trichloroacetyl-3-bromo-2-methoxyazulene, 1-trichloroacetyl-3-chloro-2-ethoxyazulene, 4,4-dimethyl-2-cyclopenten-1-one, α-methylene-γ-butyrolactone, 5,6-dihydro-2*H*-pyran-2-one, 3,3,3-trifluoro-2-hydroxy-1-phenyl-1-propanone, codeinone (an oxidative product of codeine) or morphinone (an oxidative metabolite of morphine), induced autophagic cell death characterized by vacuolization (detected by transmission electron microscopy), and the granular distribution of acridine orange and translocation of microtubule-associated protein light chain-3 fused with green fluorescent protein (LC3-GFP) into the autophagosome, and formation of autophagosom-engulfing organelle (18). If this is the case for rhinacanthin C, its activity should be abolished by treatment with

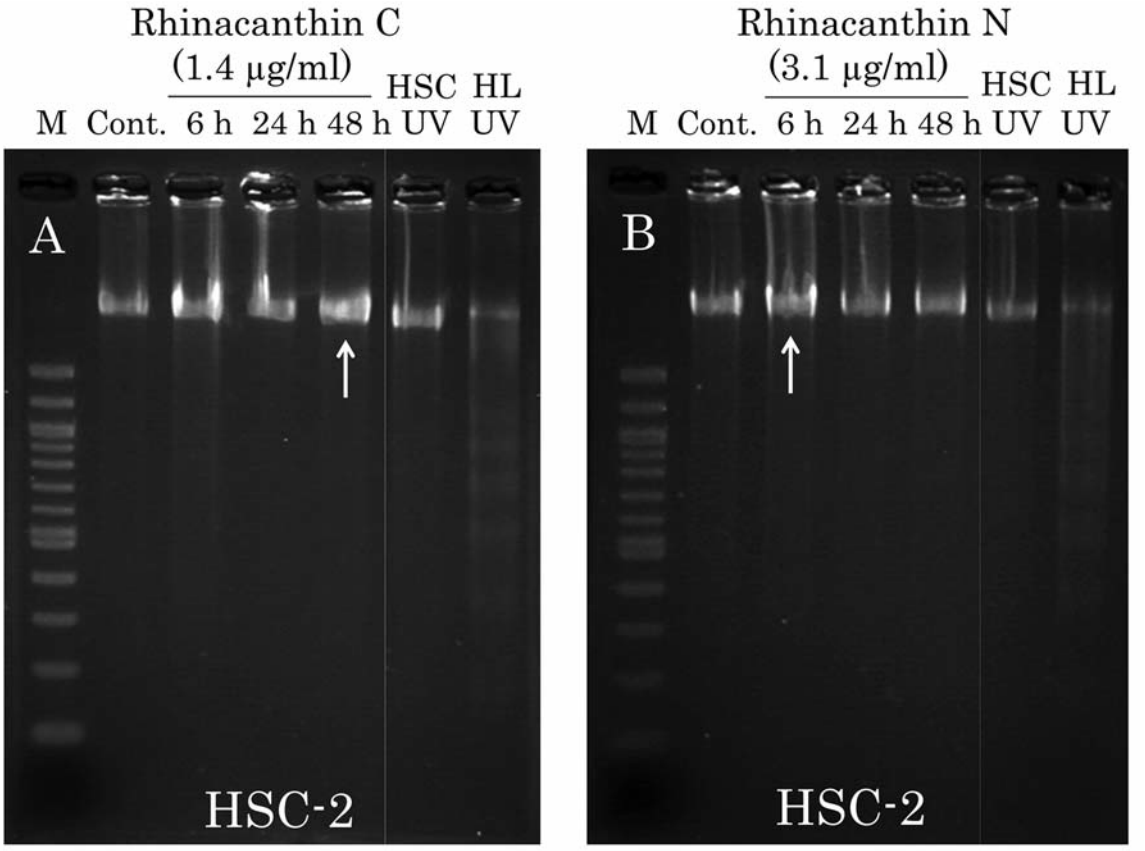

Figure 2. *Effect of rhinacanthin C and N on DNA fragmentation. HSC-2 cells were incubated for 6, 24 or 48 h with the indicated concentrations of rhinacanthin C or N, and the DNA fragmentation was assayed by agarose gel electrophoresis. M, DNA marker; Cont, Control; UV, DNA was prepared from HL-60 and HSC-2 cells that had been exposed to UV irradiation (6 J/m^2/min, 1 min) and then incubated for 3 h in regular culture medium. Arrows indicate the large DNA fragment.*

N-acetyl-L-cysteine, which interacts with the β-position of the α,β-unsaturated carbonyl moiety.

The present study also demonstrated that rhinacanthin C most potently inhibited the RANKL-stimulated osteoclastogenesis. The relatively lower cytotoxicity of rhinacanthin C against normal oral cells (HGF, HPC, HPLF) suggests its possible application in therapy of oral diseases. The signaling pathway of rhinacanthin C in various responsive cells remains to be investigated.

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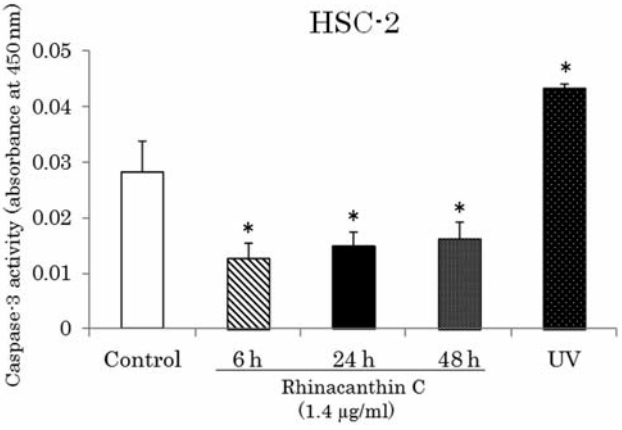

Figure 3. *Effect of rhinacanthin C on caspase-3 activity. HSC-2 cells were incubated for 6, 24 or 48 h with 1.4 μg/ml of rhinacanthin C, and the caspase-3 activity was determined by the cleavage of DEVD-pNA. UV, HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 1 min) and cultured for 3 h. Each value represents the mean±S.D. of triplicate assays. *p<0.05, compared with control values.*

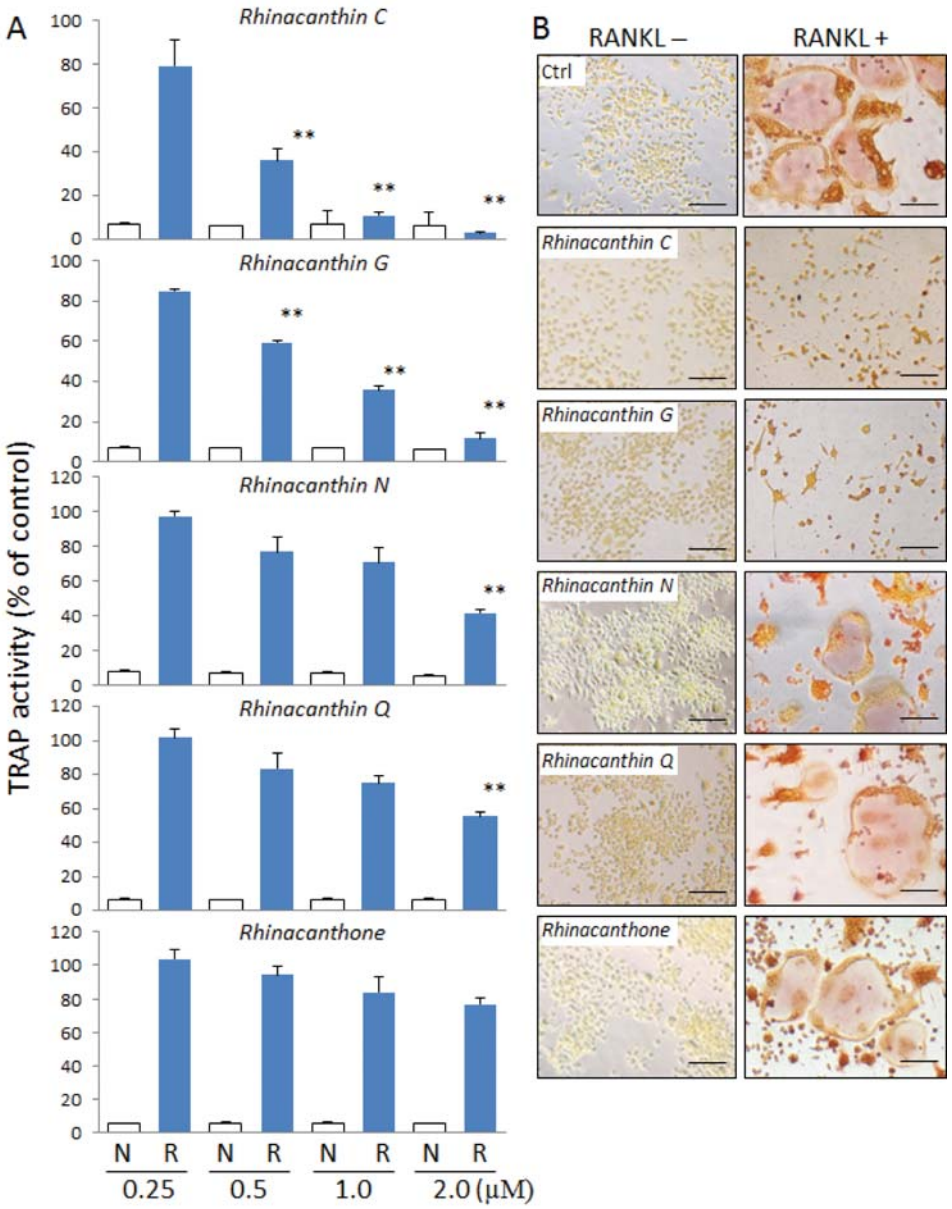

Figure 4. *Effects of isolates from methanolic extract of Rhinacanthus nasutus on osteoclast differentiation. Bone marrow-derived macrophages were cultured for four days in the presence or absence of receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) with isolated compounds. A: tartrate-resistant acid phosphatase (TRAP) activity of the medium. N, Untreated control; R, RANKL treated. B: TRAP staining, representative range of morphological appearance. All samples contained 0.2% dimethyl sulfoxide (DMSO) as a vehicle control. Data are the means±SD of quadruplicate assays. **p<0.01, compared with control. Scale bar=100 μm.*

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Received November 24, 2012
Revised December 27, 2012
Accepted January 3, 2013