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Induction of Non-apoptotic Cell Death in Human Oral Squamous Cell Carcinoma Cell Lines by *Rhinacanthus nasutus* Extract

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Abstract. *Background: We recently reported that the MeOH extract of aerial parts and root of Rhinacanthus nasutus showed diverse biological activity, with most activity being concentrated into the EtOAc layer separated by sequential organic solvent extractions. In the present study, the EtOAc extracts were further separated by silica-gel column chromatography into five fractions (Frs. 1-5), and their cytotoxicity and apoptosis-inducing activity investigated. Materials and Methods: 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 from the dose-response curve. Tumor specificity (TS) was determined by the ratio of the mean CC_{50} for normal cells to the one for tumor cell lines. DNA fragmentation was assayed by agarose gel electrophoresis. Caspase-3/-7 activation was monitored by cleavage of substrates either spectrophotometrically or by western blot analysis. Results: Among five fractions of the EtOAc extract, Fr. 1, eluted with $CHCl_3$-MeOH (50:1), showed the highest tumor specificity (TS=3.3) as compared with other fractions eluted at higher concentrations of MeOH in $CHCl_3$ (TS=1.0-2.8). Fr. 1 did not induce internucleosomal DNA fragmentation or induced only marginal level of caspase-3 activity in either human promyelocytic leukemia HL-60 cells and human oral squamous cell carcinoma (OSCC) cell lines HSC-2. Conclusion: The present study suggests that hydrophobic substances of EtOAc extract show tumor specific cytotoxicity by inducing little or no apoptosis.*

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The shrub *Rhinacanthus nasutus* (L.) Kurz (Acanthaceae) (Figure 1) is widely distributed in Southeast Asian countries and is used for the treatment of pneumonia, diabetes, hypertension and skin diseases (1). The root and aerial parts of this plant contain naphthoquinone esters such as rhinacanthins C, D, N and Q that exhibit apoptosis-inducing (2-5), antitumor, antiviral (6), antiallergy (7) and anti-inflammatory activities (8, 9), and are used for the treatment of pneumonia, diabetes, hypertension and skin diseases (1). In order to search for new biological activities of the components of this plant, we crudely separated the MeOH extract into four fractions (*n*-hexane, EtOAc, *n*-BuOH and H_2O layers) by successive organic solvent extractions and compared their biological activities (10). We found that the EtOAc extract showed the highest tumor specific cytotoxixicty, and inhibited nitric oxide production stimulated by lipopolysaccharide and the osteoclastogenesis stimulated by receptor activator for nuclear factor-κB ligand in mouse macrophage-like RAW264.7 cells (10). In the present study, we further separated the EtOAc extract into five fractions with silica-gel column chromatography using $CHCl_3$-MeOH as elution solvent, and investigated their tumor specific cytotoxicity and apoptosis inducing activity.

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 medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO), Wako Pure Chemical, Osaka, Japan; melphalan, Sigma-Aldrich Inc., St. Louis, MO, USA.

Extracts and sequential fractionation with organic solvents. 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 one of the

authors (Professor Y. Shirataki) and also a voucher specimen (#201006060) is deposited in the Medicinal Plant Garden of Josai University.

The root (1.2 kg) of *R. nasutus* was extracted three times with MeOH under reflux for 3 hours each. The MeOH extract (83 g) was fractionated by sequential organic solvent extraction as described previously (10). The EtOAc layer (16.0 g) was chromatographed on a silica-gel column with different solvent ratios of $CHCl_3$-MeOH (50:1, 20:1, 10:1, MeOH) to yield five fractions: Fr. 1 (eluted with $CHCl_3$-MeOH=50:1, 9.1 g), Fr. 2 (eluted with $CHCl_3$-MeOH=50:1, 2.4 g), Fr. 3 (eluted with $CHCl_3$-MeOH=20:1, 1.3 g), Fr. 4 (eluted with $CHCl_3$-MeOH=10:1, 1.5 g) and Fr. 5 (eluted with MeOH, 1.5 g) (Figure 2).

Cell culture. Human oral squamous cell carcinoma (OSCC) cell lines (HSC-2, HSC-3, HSC-4), human promyelocytic leukemia HL-60 cells, and normal human oral cells [gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF)] were prepared and cultured, as described previously (10). HL-60 cells were cultured at 37˚C in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under a humidified 5% CO_2 atmosphere. Other cells were cultured in 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 hours to allow cell attachment. Near-confluent cells were treated for 48 hours 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 MTT method (10). 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 (10). 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 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 hours 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). Fr. 1 (0-60 μg/ml) was applied to a 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89mM 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 hours 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 resting for 10 min on ice 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) containing substrates for caspase-3 [DEVD-*p*NA (*p*-nitroanilide)]. After incubation for 3 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured with microplate reader.

Figure 1. *The shrub Rhinacanthus nasutus (L.) Kurz (Acanthaceae).*

Assay for western blotting. HSC-2 cells were treated with 15, 30 and 60 μg/ml of Fr. 1, respectively. The cleavage of poly(ADP-ribose)polymerase (PARP) was measured using a Promega PARP (Asp 214) human specific antibody (distributed by Cell Signaling Technology, Inc. Boston, MA, US). In brief, cells were washed in ice-cold PBS, scraped, collected in lysis buffer [20 mM HEPES pH7.4, 1% Triton-X 100, 150 mM NaCl, 1.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) plus 1× protease inhibitor]. The cell lysates were applied to 8% Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) and the protein bands in the gels were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dry milk, incubated with primary antibody [anti-cleaved PARP1 (Cell Signaling Technology), anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, USA)], and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies.

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

Results

Cytotoxic activity of EtOAc fractions. Among the five fractions prepared from the EtOAc layer, Fr. 1 exhibited the highest cytotoxicity against human tumor cell line (mean CC_{50} against tumor cells=8.8 μg/ml), followed by Fr. 2 (8.9 μg/ml) , Fr. 3 (13.6 μg/ml), Fr. 4 (29.7 μg/ml) and Fr. 5 (>31.3 μg/ml) (Table I). Fr. 1 also had the highest tumor specificity (TS=3.3), followed by Fr. 2 (TS=2.8), Fr. 3 (TS=2.3), Fr. 4 (TS≥1.1) and Fr. 5 (TS≥1.0).

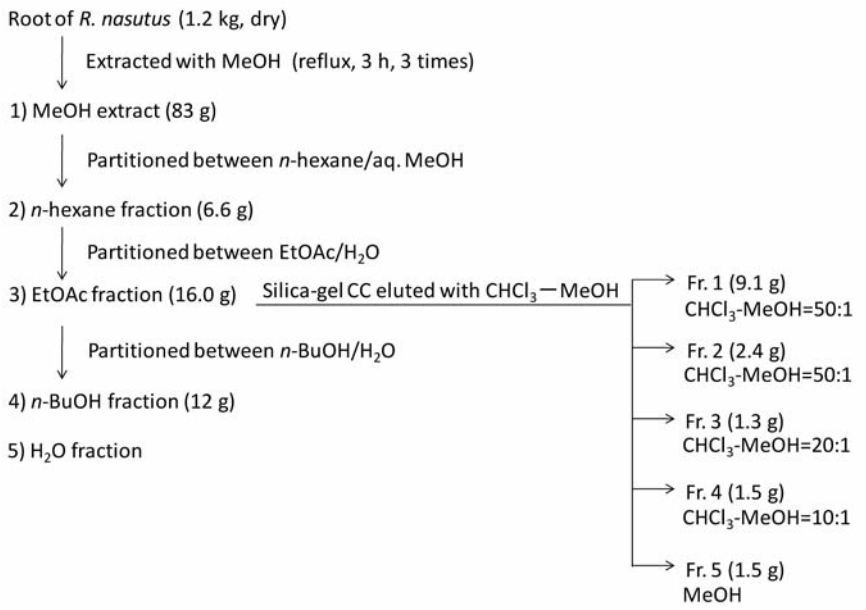

Figure 2. *The EtOAc layer (16.0 g) was chromatographed on a silica-gel column with different solvent ratios of $CHCl_3$-MeOH (50:1, 20:1, 10:1, MeOH) to yield five fractions.*

Effect on DNA fragmentation. Fr. 1 (2.5 μg/ml, approximately 1.9-fold of CC_{50}) did not induce internucleosomal DNA fragmentation in HL-60 cells. With increasing concentrations (5 and 10 μg/ml, corresponding to 3.8- and 7.7-fold of CC_{50} respectively), slight DNA fragmentation was observed, but not so markedly as observed in UV-irradiated HL-60 cells (positive control) (Figure 3A).

Fr. 1 (15, 30, 60 μg/ml, corresponding to 1.4-, 2.7- and 5.4-fold of CC_{50}) did not induce internucleosomal DNA fragmentation in HSC-2 cells. It was unexpected that even UV irradiation did not induce DNA fragmentation in HSC-2 cells (Figure 3B).

Effect on caspase activation. Treatment of HL-60 cells with Fr. 1 (1.25 μg/ml) (corresponding to 1-fold of CC_{50}) activated caspase-3 activity only slightly, but significantly (*p<0.01) (Figure 4A). However, the extent of caspase-3 activation (2.9-fold) was much less than that attained by UV irradiation (20.9-fold) (Figure 4A).

Treatment of HSC-2 cells with Fr. 1 (60 μg/ml) (corresponding to 5.4-fold of CC_{50}) did not activate caspase-3 activity. It should be noted that UV irradiation activates caspase-3 in HSC-2 cells to an extent much lower than the one in HL-60 cells (Figure 4B). Western blot analysis revealed that Fr. 1 at lower concentrations (15, 30 μg/ml) did not induce the cleavage of 116 kDa nuclear PARP in HSC-2 cells, and a higher concentration (60 μg/ml) only marginally produced cleaved PARP, as compared with that induced by UV irradiation (Figure 5).

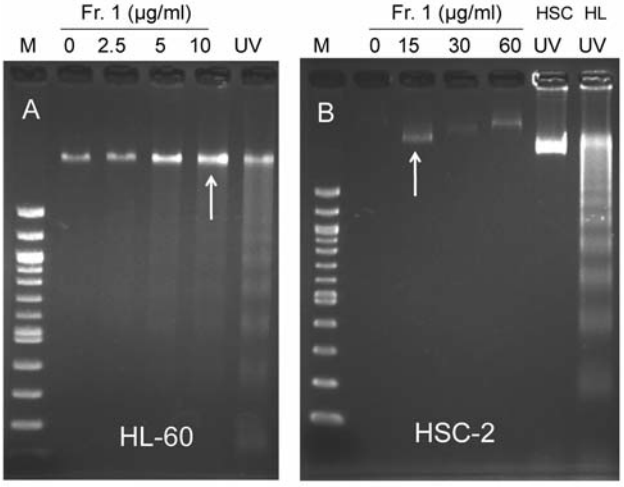

Figure 3. *Effect of Fr. 1 on DNA fragmentation. HL-60 (A) and HSC-2 (B) cells were incubated for 6 hours with the indicated concentrations of Fr. 1, and DNA fragmentation was assayed by agarose gel electrophoresis. M, DNA marker; UV, DNA was prepared from HL-60 and HSC-2 cells that had been exposed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

Discussion

The present study demonstrated that among five fractions of EtOAc extract, Fr. 1, having the most hydrophobicity, exhibited the highest levels of both cytotoxicity and tumor specificity; both cytotoxicity and tumor specificity declined

Table I. *Cytotoxic activity of Rhinacanthus nasutus extracts against human normal and tumor cells. Cells were incubated for 48 hours with various concentrations of EtOAc fractions. CC_{50}, 50% cytotoxic concentration. Data are the mean±S.D. of three independent experiments.*

	CC_{50} (μg/ml)							
	Tumor cell lines				Normal cells			
	HSC-2	HSC-3	HSC-4	HL-60	HGF	HPC	HPLF	TS
Fr. 1	11.1±7.9	2.3±0.9	21.0±0.9	1.3±0.1	27.1±4.9	30.8±0.9	>31.3	3.3
Fr. 2	5.7±1.3	5.9±4.5	20.5±4.3	3.3±0.5	20.9±8.1	22.2±1.3	30.0±1.4	2.8
Fr. 3	25.4±1.9	12.5±7.1	8.3±0.9	8.1±2.6	>31.3	30.9±0.6	>31.3	2.3
Fr. 4	>31.3	30.3±1.7	29.0±2.0	28.2±4.1	>31.3	>31.3	>31.3	>1.1
Fr. 5	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>31.3	>1.0

HGF: Human gingival fibroblasts; HPC: human pulp cells; HPLF: human periodontal ligament fibroblast; TS, tumor specificity index.

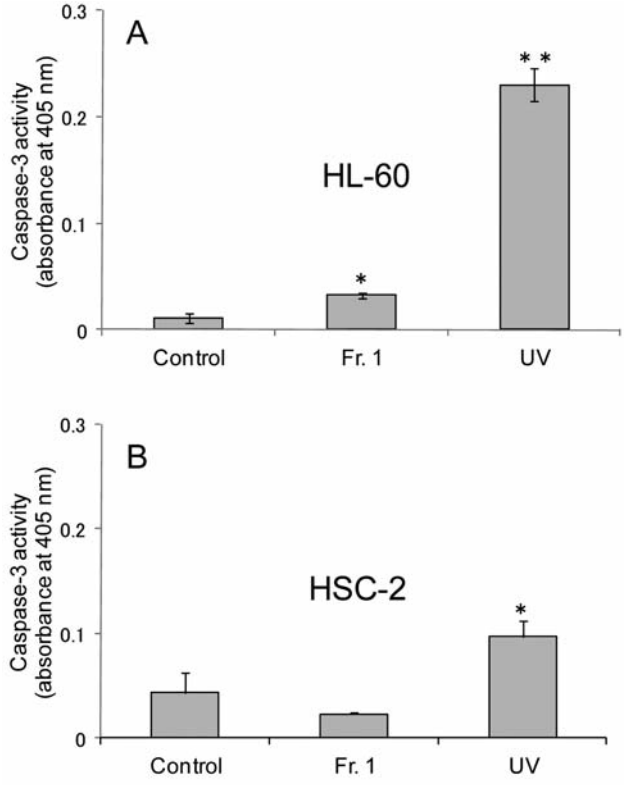

Figure 4. *Effect of Fr. 1 on caspase-3 activity. HL-60 (A) and HSC-2 (B) cells were incubated for 6 hours with 1.25 or 60 μg/ml of Fr. 1, respectively and the caspase-3 activity was determined by the cleavage of DEVD-pNA. UV; HL-60 and HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 1 min) and cultured for 3 hours. Each value represents the mean±S.D. of triplicate assays. *p<0.01, *p<0.05 and **p<0.001 compared to control values.*

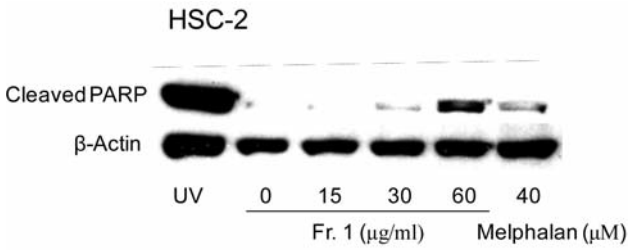

Figure 5. *Activation of caspase-3/-7 by Fr. 1. HSC-2 cells were incubated for 6 hours with the indicated concentrations of Fr. 1, and the production of cleaved poly(ADP-ribose)polymerase (PARP) was detected by western blot analysis. UV, HSC-2 cells were exposed to UV irradiation (6 J/m^2/min, 3 min) and cultured for 3 hours. Melphalan, a well-known anti-tumor agent, was used as positive control.*

with decreasing hydrophobicity (Fr. 2>Fr. 3>Fr. 4>Fr. 5). Further purification of this fraction is underway.

The present study also demonstrated that Fr. 1 induced apoptosis of HL-60 cells very weakly, based on the only marginal levels of induction of internucleosomal DNA fragmentation (Figure 3A) and caspase-3 activation (Figure 4A), as compared to UV irradiation. On the other hand, Fr. 1 did not induce these apoptosis markers in HSC-2 cells (Figures 3B and 4B). We also observed under light microscopy that Fr. 1 did not induce the appearance of apoptotic body that may be quantifiable as sub-G1 population by flow cytometry (data not shown). It was unexpected that Fr. 1 would induce the cleavage of PARP, albeit to a much lesser extent than that attained by UV irradiation in HSC-2 cells (Figure 5) and at a high concentration. One may note that while in some cases PARP cleavage can be initiated by caspase-3 activation during apoptosis (11-13), the effect of Fr. 1 on PARP1 cleavage is brought about by mechanisms other than caspase activation.

We found that whether cells are committed to apoptosis or not was dependant upon on the type of cells. Upon UV exposure, HL-60 cells (Figure 3A and 4A), but not HSC-2 cells (Figures 3B and 4B), responded with apoptosis. Furthermore, the treatment of HSC-2 cells with even

melphalan, a popular alkylating agent (14), stimulated PARP cleavage to a much lesser extent (Figure 5). This suggests that the apoptosis-inducing machinery may not be normally functioning in HSC-2 cells. It should be noted that Fr. 1 cleaved DNA to produce large fragments in both HL-60 and HSC-2 cells (indicated by arrows in Figure 3A and 3B), suggesting the activation of DNase(s) other than caspase-activated DNase (15). Further experiments are required to confirm this point.

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