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 (CC50) was determined from the dose-response curve. Tumor specificity (TS) was determined by the ratio of the mean CC50 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 CHCl3-MeOH (50:1), showed the highest tumor specificity (TS=3.3) as compared with other fractions eluted at higher concentrations of MeOH in CHCl3 (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.

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 CHCl₃-MeOH (50:1, 20:1, 10:1, MeOH) to yield five fractions: Fr. 1 (eluted with CHCl₃-MeOH=50:1, 9.1 g), Fr. 2 (eluted with CHCl₃-MeOH=50:1, 2.4 g), Fr. 3 (eluted with CHCl₃-MeOH=20:1, 1.3 g), Fr. 4 (eluted with CHCl₃-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% CO₂ atmosphere. Other cells were cultured in DMEM supplemented with 10% FBS and antibiotics.

**Assay for cytotoxic activity.** The cells (3 × 10³ 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 attachment. Near-confluent cells were treated for 48 hours with CHCl₃-MeOH=10:1, 1.5 g) and Fr. 5 (eluted with MeOH, 1.5 g) (Figure 2).

**Figure 1. The shrub Rhinacanthus nasutus (L.) Kurz (Acanthaceae).**
Effect on DNA fragmentation. Fr. 1 (2.5 μg/ml, approximately 1.9-fold of CC50) 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 CC50 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 CC50) 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 CC50) 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 CC50) 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).

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...
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 PARP 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

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**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. CC50, 50% cytotoxic concentration. Data are the mean±S.D. of three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Fr. 1</th>
<th>Fr. 2</th>
<th>Fr. 3</th>
<th>Fr. 4</th>
<th>Fr. 5</th>
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<td>HSC-2</td>
<td>11.1±7.9</td>
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<td>&gt;31.3</td>
<td>&gt;31.3</td>
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<tr>
<td>HSC-3</td>
<td>2.3±0.9</td>
<td>5.9±4.5</td>
<td>12.5±7.1</td>
<td>30.3±1.7</td>
<td>&gt;31.3</td>
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<tr>
<td>HSC-4</td>
<td>21.0±0.9</td>
<td>20.5±4.3</td>
<td>8.3±0.9</td>
<td>29.0±2.0</td>
<td>&gt;31.3</td>
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<tr>
<td>HL-60</td>
<td>1.3±0.1</td>
<td>3.3±0.5</td>
<td>8.1±2.6</td>
<td>8.2±4.1</td>
<td>&gt;31.3</td>
</tr>
<tr>
<td>HGF</td>
<td>27.1±4.9</td>
<td>20.9±8.1</td>
<td>&gt;31.3</td>
<td>&gt;31.3</td>
<td>&gt;31.3</td>
</tr>
<tr>
<td>HPC</td>
<td>30.8±0.9</td>
<td>22.2±1.3</td>
<td>&gt;30.9±0.6</td>
<td>&gt;31.3</td>
<td>&gt;31.3</td>
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<tr>
<td>HPLF</td>
<td>30.8±0.9</td>
<td>30.0±1.4</td>
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</tbody>
</table>

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


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