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Identification of 6-methylsulfinylhexyl isothiocyanate as an apoptosis-inducing component in wasabi

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Abstract

The ethanol extract from Japanese horseradish wasabi was found to inhibit cell proliferation in human monoblastic leukemia U937 cells by inducing apoptotic cell death. Separation by methods including silica gel chromatography and preparative HPLC gave an active compound, which was identified as 6-methylsulfinylhexyl isothiocyanate (6-HITC). Several lines of evidence indicated that 6-HITC induced apoptosis in U937 cells and human stomach cancer MKN45 cells. Thus, 6-HITC is potentially useful as a natural anti-cancer agent.

Keywords: *Wasabia japonica*; Brassicaceae; Wasabi; Apoptosis; 6-Methylsulfinylhexyl isothiocyanate; U937 cell; MKN45 cell

1. Introduction

Several chemotherapeutic compounds have been reported to induce apoptosis, which may be a primary mechanism of their anti-cancer activity (Gunji et al., 1991; Skladanowski and Konopa, 1993).We previously reported that epigallocatechin gallate, a major green tea component, and other polyphenolic compounds from tea induce apoptosis in human monoblastic leukemia U937 cells and human stomach cancer MKN45 cells (Saeki et al., 1999, 2000a,b). Several other polyphenolic compounds soluble in ethanol are also known to exhibit this activity (Surh, 1999; Galati et al., 2000; Romero et al., 2002; Mouria et al., 2002; Rafi et al., 2002).

During the course of a search for naturally occurring compounds with apoptosis-inducing activity, we found that an ethanol extract of Japanese horseradish wasabi (*Wasabia japonica*) contains active compounds. In the present work, we identified one of them as 6-methylsulfinylhexyl isothiocyanate.

2. Results and discussion

The wasabi ethanol extract inhibited cell growth of human monoblastic leukemia U937 cells (Fig. 1). To identify an active component in the ethanol extract, the latter was dissolved in hexane and the soluble fraction was then fractionated by silica gel chromatography. Successive elution with hexane, hexane/ethanol (9:1), and ethanol yielded three fractions in yields of 7.2, 42.2, and 22.3% (w/w), respectively. Each fraction was dried and dissolved in ethanol to evaluate their inhibitory activity against cell proliferation. Upon treatment with fractions at 50 μg/ml eluted with hexane, hexane/ethanol (9:1), and ethanol the values of the cell number relative to untreated cells (100%) after incubation for 16 h were 62, 28, and 24%, respectively. The hexane/ethanol (9:1) fraction gave a relatively small number of peaks on HPLC (Fig. 2), and repeated HPLC of this fraction gave a pure compound termed Compound **1** with a retention time of about 6.8 min. The yield was about 5 mg from 100 g of wasabi roots.

The spectrometric data for Compound **1** were as follows: colorless liquid; ^{1}H NMR (CD_3OD, 400 MHz) δ 3.57 (2H, *t*, $J=6.4$ Hz, H-1), 2.82 (2H, *td*, $J=13.0$, 7.0 Hz, H-6), 2.63 (3H, *s*, CH_3SO), 1.6–1.8 (4H, *m*, H-2 and

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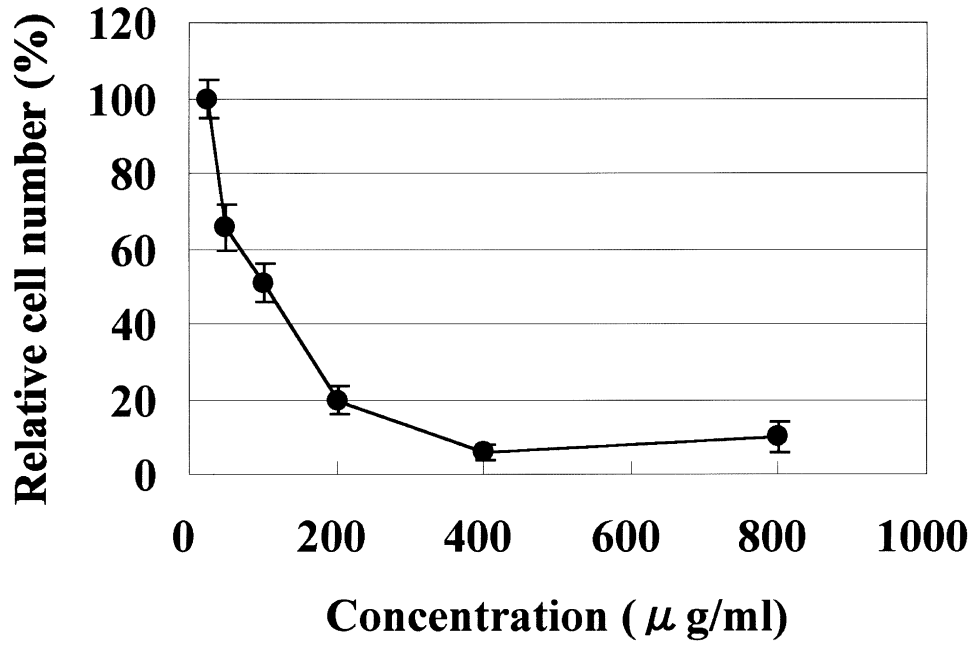

Fig. 1. Effects of the wasabi ethanol extract on proliferation of U937 cells. The extract was dissolved in ethanol, and an aliquot (1 μl) of the solution was added to 200 μl of culture medium containing 2×10^4 cells to give final concentrations as indicated. After incubation for 16 h, an Alamer blue solution (20 μl) was added and fluorescence was determined after 2 h with excitation at 560 nm and emission at 590 nm. The relative cell number was calculated in comparison with the control cells (in 200 μl) that had received ethanol only (1 μl). Each point represents the average of three determinations.

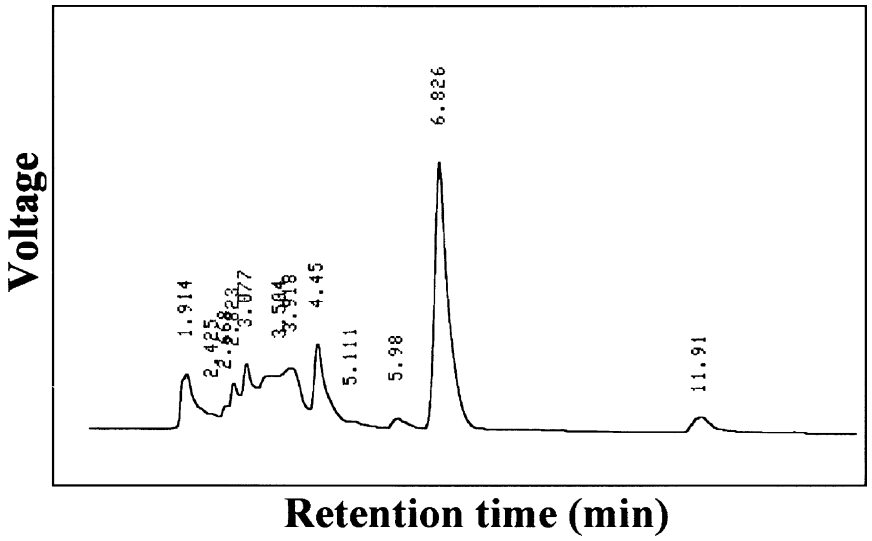

Fig. 2. HPLC of a fraction obtained by elution with hexane–ethanol (9:1) on silica gel column chromatography of the acetone-soluble fraction of the wasabi ethanol extract. HPLC was carried out using a column of Shiseido Capcell Pak C18 (4.6×150 mm) and elution with 40% methanol in H_2O. The flow rate was 1 ml/min and detection was at 205 nm.

H-5), 1.4–1.6 (4H, *m*, H-3 and H-4); ^{13}C NMR (CD_3OD, 100 MHz) δ 131.0 (N*C*S), 54.6 (C-1), 45.8 (C-6), 38.1 (*C*H_3SO), 30.7 (C-2), 28.8 (C-5), 27.2 (C-3), 23.4 (C-4); FABMS *m*/*z* 150 $[M+H]^+$. These data identified Compound **1** as 6-HITC, CH_3–SO–$(CH_2)_6$–N=C=S, on the basis of reported data (Morimitsu et al., 2002).

When U937 cells were incubated for 16 h with Compound 1 identified here as 6-HITC, the cell numbers were reduced to 47.3, 24.0, and 13.5% of the control at concentrations of 2, 4, and 8 μg/ml, respectively. As shown in Fig. 3, 6-HITC induced formation of apoptotic bodies in U937 cells, suggesting that the growth inhibition may be due to its apoptosis-inducing activity.

To test this possibility, induction of DNA fragmentation and its inhibition by the caspase inhibitor (Mashima et al., 1995) were examined, since DNA ladder formation is one of the characteristics of apoptosis (Sellins and Cohen, 1987; Gunji et al., 1991) and since

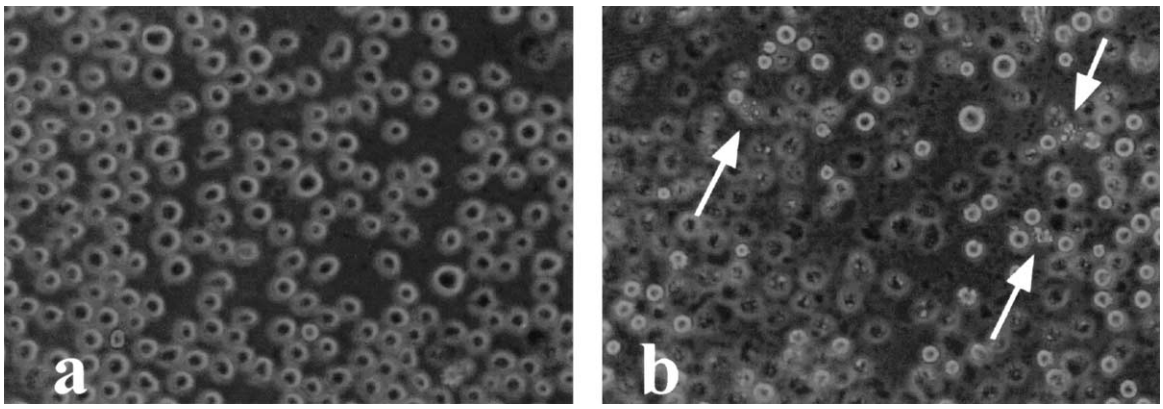

Fig. 3. Morphological differences between control U937 cells (a) and the cells treated with isolated 6-HITC at 2 μg/ml for 16 h (b). Apoptotic bodies are indicated by arrows.

caspases are essential proteases in the execution of apoptosis (Mashima et al., 1995; Nagata, 1997; Cohen, 1997). The results of agarose gel electrophoresis of DNA extracted from the treated cells revealed that isolated 6-HITC induced DNA fragmentation in a nucleosome unit (Fig. 4) and that this DNA fragmentation was inhibited by the pan-caspase inhibitor Z-Asp-CH_2-DCB (Fig. 4). These findings suggested that 6-HITC caused apoptosis in U937 cells.

When 6-HITC-treated cells were stained with Hoechst 33342, chromatin condensation was observed (Fig. 5). The pecentages of apoptotic cells with chromatin condensation were 4.6, 12.0, and 18.2% of the total viable cells in the cells treated for 16 h with 6-HITC at concentrations of 2, 4, and 8 μg/ml, respectively. Chromatin condensation is also one of the features characteristic of apoptosis (Darzynkiewicz et al., 1994).

In addition to human monoblastic leukemia U937 cells, 6-HITC isolated here induced apoptosis in human stomach cancer MKN45 cells as evidenced by the DNA ladder formation in a dose-dependent manner (Fig. 6) and by chromatin condensation (Fig. 7). The DNA fragmentation was inhibited by the presence of caspase inhibitor *Z*-Asp-CH_2-DCB (Fig. 6). These results indicate that 6-HITC is an apoptosis inducer.

Ono et al. (1998) previously isolated and identified 6-HITC from wasabi as an antibacterial compound. Moreover, isothiocyanate compounds including allyl isothiocyanate and sulforaphane, a homologue of 6-HITC, are known to induce apoptosis (Gamet-Payrastre et al., 2000; Chiao, 2002; Fimognari, 2002; Thornalley, 2002). Allyl isothiocyanate is a component of wasabi and the present study adds a new member of the apoptosis-inducing components in wasabi.

Morimitsu et al. (2002) reported recently that 6-HITC induces phase-II detoxification enzymes such as glutathione *S*-transferases, suggesting its usefulness as a chemopreventive agent. They also reported that 6-HITC is an inhibitor of platelet aggregation and that 6-HITC has potential anti-cancer activity (Morimitsu et al., 2000). The induction of a quinone oxidoreductase and its mRNA expression by 6-HITC has also been implicated in cancer chemoprevention (Hou et al., 2000). Yano et al. (2000) reported that 6-HITC inhibits the development of lung tumors in mice treated with a chemical carcinogen due to the suppression of the initiation stage. Fuke et al. (1997) reported the inhibitory effect of 6-HITC on the growth of human stomach tumor cells and on skin carcinogenesis of mice induced by a phorbol ester. The present findings are consistent with evidence presented orally by Nakamura and Fuke (2000) that 6-HITC induces apoptosis in several human leukemic cell lines.

The finding that three fractions separated by silica gel chromatography exhibited more or less inhibitory activity against U937 cell proliferation suggests the possibility that wasabi contains several unidentified compounds with apoptosis-inducing activity. Indeed, on the basis of the concentration of 6-HITC in the ethanol extract (4.8 mg/ml) as determined by HPLC, we estimated that about 60% of the total activity in the wasabi ethanol extract could be accounted for by 6-HITC. It is known that wasabi contains other isothiocyanates such as 7-methylthioheptyl isothiocyanate and 8-methylthiooctyl isothiocyanate (Ina et al., 1989), and these isothiocyanates are also expected to contribute to the apoptosis-inducing activity in the ethanol extract. The identification of the active compounds awaits future investigations.

Wasabi is popularly used as a pungent spice in Japan. Apoptosis induction by 6-HITC in human stomach cancer MKN45 cells suggests that frequent and/or high ingestion of wasabi in a diet may contribute to reduce the risk of stomach cancer. 6-HITC deserves further investigation as a vegetable-derived chemopreventive agent.

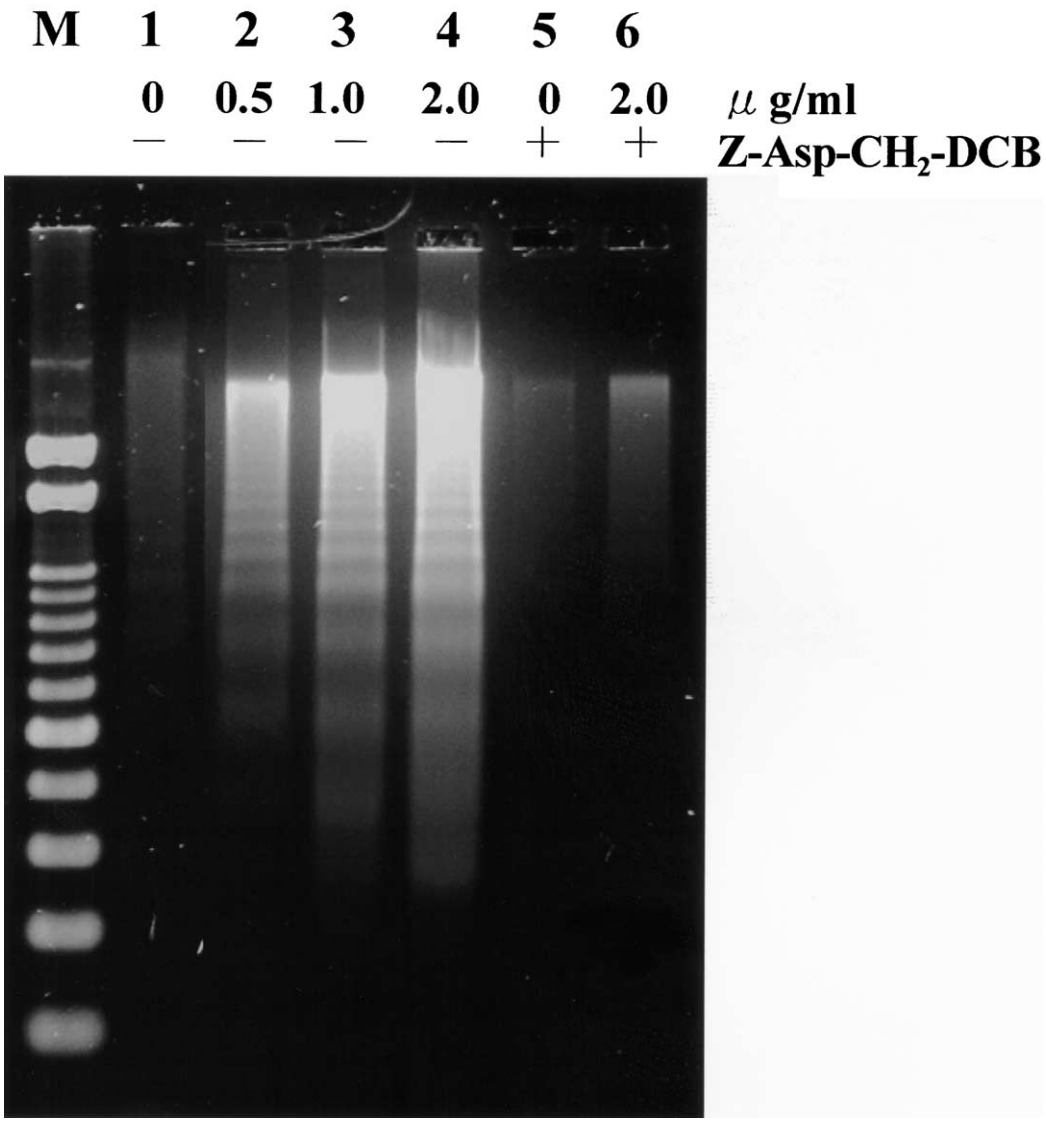

Fig. 4. Fragmentation of DNA from U937 cells treated with 6-HITC. U937 cells were incubated at 37 °C for 16 h with 6-HITC at the concentrations indicated (lanes 1–6). DNA isolated from the cell pellets was then electrophoresed in 2% agarose gel, stained with SYBR Green I, and imaged using FluoroImager. The fragmentation of DNA in the presence (+) of the caspase inhibitor Z-Asp-CH_2-DCB at 200 μM was also examined.

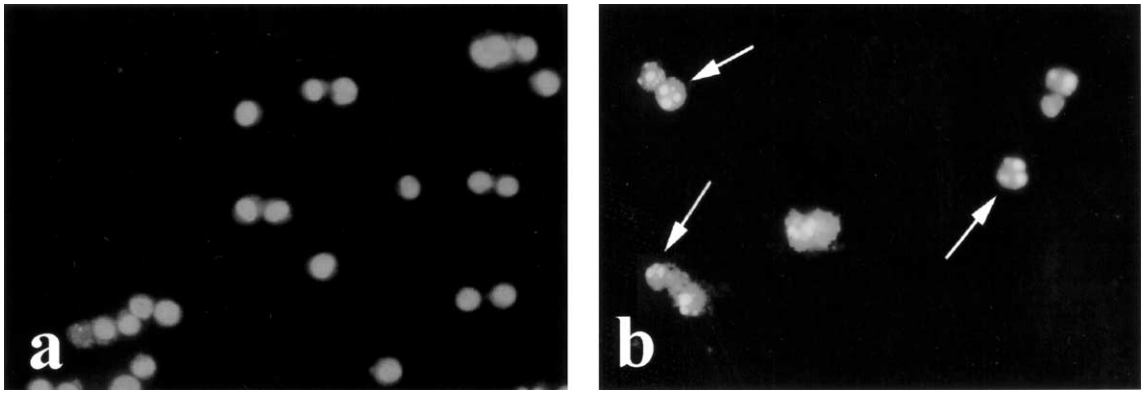

Fig. 5. Hoechst 33342 staining of U937 cells. The cells treated with isolated 6-HITC were fixed with 1%-glutaraldehyde, stained with Hoechst 33342 and examined under a fluorescence microscope with excitation at 330–380 nm. Chromatin condensation as indicated by arrows was observed for the cells treated with 6-HITC at 2 μg/ml (b). Untreated control cells are shown in (a).

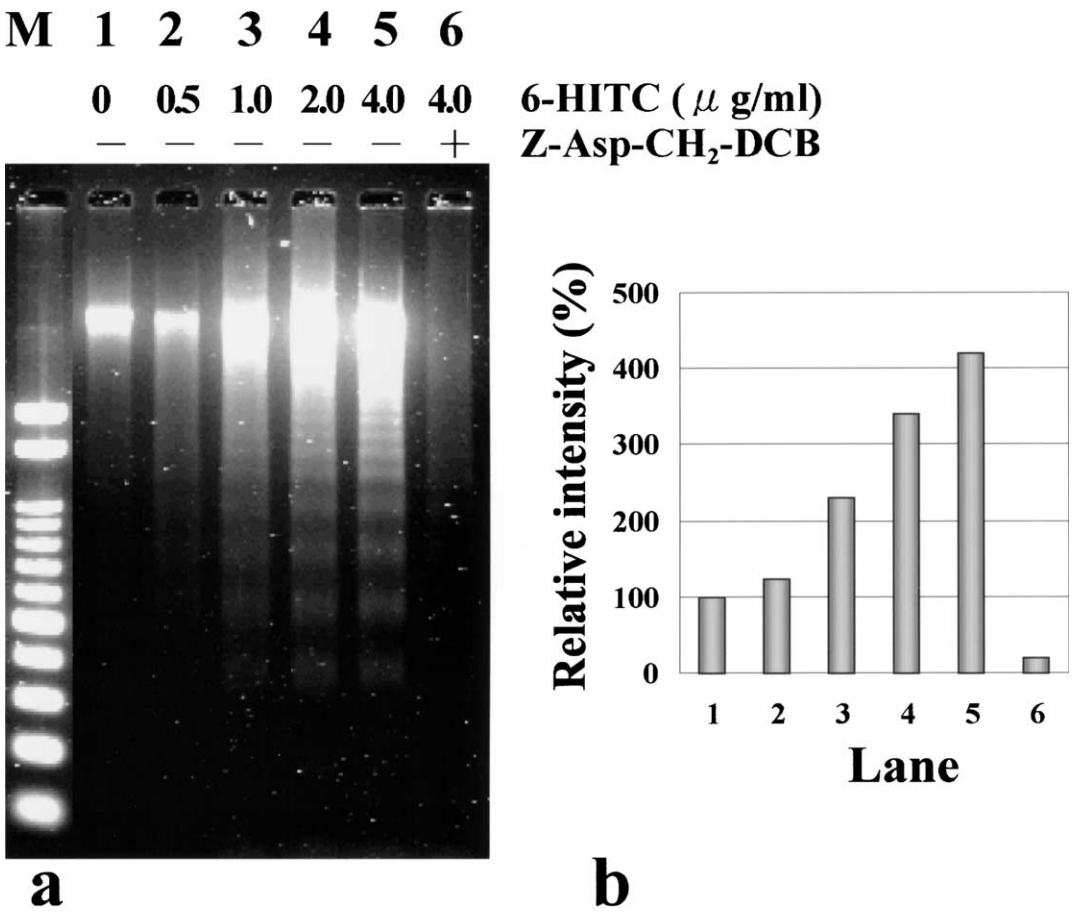

Fig. 6. Dose-dependent DNA fragmentation induced by 6-HITC. (a) MKN45 cells were incubated at 37 °C for 16 h with isolated 6-HITC at the concentrations indicated with (+) or without (−) the caspase inhibitor Z-Asp-CH_2-DCB at 200 μM (lanes 1–6). M, size marker DNA. (b) The degrees of DNA fragmentation in (a) are expressed in terms of fluorescence intensity relative to that of the control (lane 1) as determined using FluorImager.

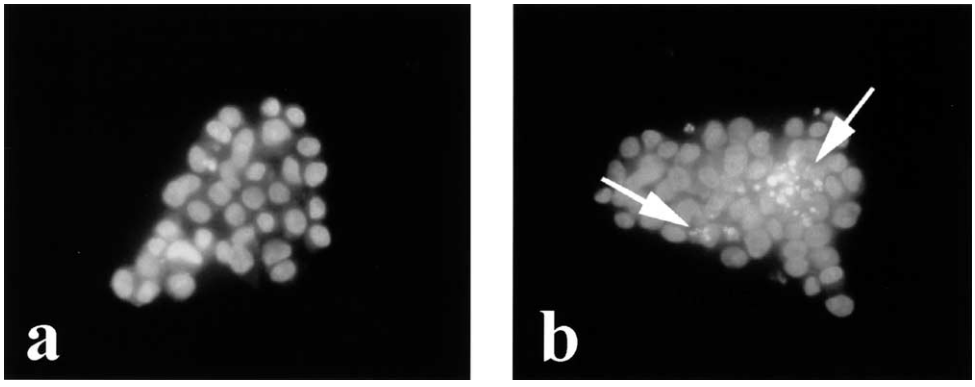

Fig. 7. Hoechst 33342 staining of MKN45 cells. The cells treated with isolated 6-HITC were fixed with 1%-glutaraldehyde, stained with Hoechst 33342 and examined under a fluorescence microscope with excitation at 330–380 nm. Chromatin condensation as indicated by arrows was observed for the cells treated with isolated 6-HITC at 2 μg/ml (b). Untreated control cells are shown in (a).

3. Experimental

3.1. Materials

U937 cells and MKN45 cells were obtained from the Health Service Research Resources Bank, Osaka, Japan, and cultured in 10% fetal bovine serum in RPMI 1640 medium (Iwaki Glass Co. Ltd., Chiba, Japan) with 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml gentamycin at 37 °C under 5% CO_2. A caspase inhibitor Z-Asp-CH_2-DCB was obtained from Peptide Institute, Inc., Osaka, Japan.

Hoechst 33342 (bisbenzimide H 33342 Fluorochrome) was obtained from Calbiochem-Novabiochem Co., CA, USA. SYBR Green I was obtained from Molecular Probes, Inc., OR, USA.

3.2. Fractionation of an ethanol extract of wasabi

Wasabi roots were homogenized in water and the mixture was freeze-dried. An ethanol extract was separated into three fractions by silica gel chromatography. The fraction eluted with hexane–ethanol (9:1) was then separated by HPLC using a Shiseido Capcell Pak C18 column (4.6×150 mm) and eluted with 40% methanol in H_2O. By repeating this procedure we obtained a pure compound termed Compound **1**.

3.3. Chemical structure

The chemical structure of Compound **1** was determined on the basis of ^1H and ^{13}C NMR spectra using a JEOL α-400 spectrometer with tetramethylsilane as an internal standard and FAB mass spectra using a JEOL JMS-700 spectrometer.

3.4. Cell proliferation

Cells (2×10^4) in 200 μl of the culture medium were seeded in a 48 well microculture plate and incubated with or without a test sample solution in the culture medium. After 16 h, the number of viable cells was determined by the Alamar blue assay and the values were compared with control as described previously (Saeki et al., 1999, 2000a,b).

3.5. DNA fragmentation

Cells ($1–2\times10^5$) were incubated with a test sample solution for 16 h and the cells were pelleted by centrifugation. DNA isolated from the cell pellets according to the method described by Sellins and Cohen (1987) was electrophoresed in 2% agarose gel, stained with SYBR Green I, and then imaged by using FluoroImager (Molecular Dynamics Japan, Inc., Tokyo, Japan) as described previously (Saeki et al., 1999, 2000a,b). For confirmation of caspase-dependent DNA ladder formation, the caspase inhibitor Z-Asp-CH_2-DCB (Mashima et al., 1995) was included in the culture medium and incubated as described above.

3.6. Chromatin condensation

Cells incubated in the presence or absence of a test sample solution at 37 °C for 16 h were pelleted by centrifugation and washed with phosphate-buffered saline, pH 7.4. After centrifugation, the cells were fixed with 1%-glutaraldehyde at 4 °C for 2 h, and stained with Hoechst 33342 to examine chromatin condensation under a fluorescence microscope with excitation at 330–380 nm as described previously (Saeki et al., 2000b). The percentage of apoptotic cells with chromatin condensation was calculated from the number of these cells and the total viable cells determined by the Trypan blue dye exclusion assay (Isemura et al., 1993).

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