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Isolation of cytotoxic component from *Trichocolea hatcheri*

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SUMMARY

Methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**) has been identified from the New Zealand liverwort *Trichocolea hatcheri (T. hatcheri)*. This compound was tested for its growth inhibitory effects against tumor cell lines using two different assays, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay and the sulforhodamine B protein (SRB) assay. This compound showed inhibitory activity *in vitro* in the micromolar concentration range against tumor cells. These results suggest that methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**) possessed antitumorous agent.

Key words: Inhibitory effect, Tumor cells, Methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyl)oxy]-3-hydroxybenzoate, Cytotoxic activity, MTT assay, SRB assay

INTRODUCTION

An extract of *T. hatcheri* showed cytotoxic effects (P 388>150 μg / disk) against monkey kidney (BSC) cell lines. Si-gel column chromatography spread cytotoxic activity across ethyl acetate - cyclohexane 3 : 97, 5 : 95, 7 : 93 and 10 : 90 fractions containing geranyl phenyl ethers (Baek *et al.*, 1998). These compounds were obtained pure in quantities, but too small for biological assays, synthesis and biological activity report here. We recently reported that geranyl phenyl ethers showed the growth-inhibitory activity against KB (ATCC No, OCL7) cell lines using two different assays, the MTT assay and the SRB assay. These compounds showed inhibitory activity *in vitro* in the micromolar concentration range against KB cell lines (Han *et al.*, 2000 and 2001). In the present study, we investigated inhibitory effects of methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**) against tumor cell lines. The effects of adriamycin (**2**) and 5-fluorouracil (**3**) were also examined for comparison.

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MATERIALS AND METHODS

All solvents were distilled before use and were removed by rotary evaporation at temperature up to 40°C. Octadecyl-functionalized Si-gel (Aldrich) was used for reversed-phase flash chromatography, and Davisil, 35-70 μm, 150°A, was used for Si gel flash chromatography. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254} visualized with a UV lamp, then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH), and heating. MS, UV and IR spectra were recorded on Kratos MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FT-IR instruments respectively. NMR spectra, of $CDCl_3$ solutions at 25°C, were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C on a Varian VXR-300 spectrometer. Chemical shifts are given in parts per million on the δ scale referenced to the solvent peak $CHCl_3$ at 7.27 and $CDCl_3$ at 77.08.

Materials

5-Fluorouracil (**3**), adriamycin (**2**), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide, fetal

bovine serum (FBS), sulforhodamine B protein, streptomycin, and penicillin were obtained from Sigma Chemical Co. Ltd. (St. Louis, USA). Geranyl bromide, sodium hydride, and dimethylsulftate were purchased from Aldrich Chemical Co. Ltd. (Milwaukee, U.S.A). 3-Methyl-4-hydroxybenzoic acid was obtained from Merck Chemical Co. (Germany). Tumor cells were obtained from Korean Cell Line Bank in the Seoul National University. All other chemicals were of reagent grade.

Cell culture

KB, SK-MEL-3, P388 and NIH 3T3 cells were grown at 37°C in RPMI medium supplemented with 10% FBS, penicillin (100 units / ml) and streptomycin (100 μg / ml). The cells were grown in a humidified atmosphere of 95% air / 5% CO_2. Cells were dissociated with 0.25% trypsin and were counted using a Hemacytometer just before transferring them for the experiment.

Plant Material and Isolation

T. hatcheri was collected from a steep earth bank in the Morrisons Creek area, Dunedin, New Zealand, in Februrary 1996 [University of Otago Herbarium (OTA) specimen no, 048094]. It was air dried (30°C). The dry plant (13.2 g of roots stems and leaves) was ground in a blender and extracted with ethanol (600 ml) and chloroform (200 ml) and filtered. The filtered extract was rotary evaporated to a green gum (0.443 g). This extract was separated by the use of the cytotoxic activity as an isolation-guide. It was chromatographed on C18 silica gel with H_2O : CH_3CN and CH_3CN : $CHCl_3$, the active fraction of 32-1-6 (86 mg) was then applied to silica gel column chromatography. The solvent of ethyl acetate - *n*-hexane, 20 : 80 eluted active fraction 32-4-5 (13.5 mg), which showed major dark grey spots on TLC. This fraction contained one main UV-active compound by TLC. After silica gel thin-layer chromatography of 13.5 mg of fraction 32-4-5, eluted with ethyl acetate - *n*-hexane, 20 : 80, was separated to compound **1** (4.5 mg). This compound (**1**) was identified by comparing its spectral data (TLC, MS, NMR and IR) with those published or by directly comparing it with an anthentic sample (Baek *et al.*, 1998; May, 1995).

Methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**): Colorless oil; ^{1}H-NMR spectrum and EIMS match those reported previously (Perry *et al.*, 1996). Methyl-3,4- dihydroxybenzoate (278 mg, 1.65 mmol); geranyl bromide (434 mg, 2.00 mmol); NaH (60%, 66 MG, 1.65 mmol) in dry DMF (2 ml); 0; 17 hr; flash chromatography (5% EtOAC - hexane) gave 3 (220 mg, 44%). Methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**) was identified by comparing its spectral data (TLC, MS, NMR and IR) with those published or by directly comparing it with an anthentic sample (Baek *et al.*, 1998; May, 1995).

Evaluation of antitumor activity

The antitumor activities of methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**), adriamycin (**2**) and 5-fluorouracil (**3**) were determined by the modifiication of the literature methods (Mosmann, 1983; Carmichael *et al.*, 1987; Skehan *et al.*, 1987). All experimental data were expressed as the mean ±S.D. of triplicate experiments.

4,5-Dimethylthiazol-2-yl-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay

The assay is dependent on the cellular reduction of water-soluble MTT (Sigma Chemical Co. St. Louis, M.O.) by mitochondrial dehydrogenase of vial cells to a blue water-insoluble formazan crystal product which can be measured spectrophotometrically (Mosmann, 1983; Carmichael *et al.*, 1987). P388 cell lines were cultured in RPMI-1640 medium (Gubco Laboratories) containing 10% fetal bovine serum. Exponentially growing tumor cells (5×10^5) were cultured for 48 h at 37°C in a humidified 5% CO_2 incubator in the presence or absence of sample.

Sorphodamine B protein (SRB) assay

The SRB assay was performed essentially according to the method of Skehan *et al.* (1990). The methods of plating and incubation of cells were indentical to those of the MTT assay.

Evaluation of toxicity : Cytotoxicity assay

In order to determine the cytotoxicity mediated by methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**), adriamycin (**2**) and 5-fluorouracil (**3**), the colorimetric assay was used. These compounds were serially diluted in EMEM (Eagle's minimum essential medium) with 10% FBS and mixed with

equal volume of NIH 3T3 fibroblasts (5×10^4 cells / ml). After one hour, fresh culture medium was supplied to a total volume of 1~100 μM. On the third day of incubation at 37°C an incubator MTT terazolium dye (5 mg / ml; 20 μl / well; polyscience, Inc. Warrigton, PA) was added to the cells. After 3 hr, the absorbance was measured at 540 nm using ELISA reader. All experimental data were expressed as the mean ±S.D. of triplicate experiments. The 50% cytotoxic dose (CD_{50}) was calculated using the computer program.

Morphology

Changes in morphology of KB, P388 and SK-MEL-3 cells cultured in a medium with methyl-4 -[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**), adriamycin (**2**) and 5-fluorouracil (**3**) were documented by microphotography.

Statistical analysis

All values, expressed as the mean ±S.D. were statistically analyzed through analysis of Student's t-test. The P value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

The aim of our research program is to fine new classes of bioactive natural products. Time spent isolating known bioactive compounds, even ones as potent as this liverwort, is time lost. Therefore, we use a rapid "chemical screening" technique to provide a chromatographic profile of the bioactivity of extracts detected in our screens. This profile can be compared with the chromatographic behaviour of known bioactive compounds from *T. hatcheri*. The chromatographic profile can also be used to select the best solvents for a bulk extraction and the best separation methods (Cardellina *et al.*, 1993). Extracts were prepared by first drying the plant material then extracting with ethanol. This solvent will extract compounds with a wide range of polarities and is a reasonable compromise when only one solvent is used (Farnsworth, 1990). Protocols, including that of Calder *et al.* (1986), Which make use of heated organic solvents can cause artifact formation (especially methanolysis if methanol is used).

A portion of the plant extract (0.443 g) was absorbed on octadecylfunctionalized silica gel (1.0 g, Aldrich Cat. 37, 763-5) by slurrying the absorbent in a solution of the extract in EtOH - H_2O (Ca 1 ml, mainly EtOH) and removing the solvent under reduced pressure. This was then loaded on a cotton wool pad at the top of a column of the C_{18}-silica gel (10 g, 8×2 cm) which had been dry-packed and then washed successively with CH_3CN, CH_3CN - H_2O (1 : 1), and H_2O (each ca. 20 ml). The column was developed as shown in the Fig. 1. octadecyl functionalized silica gel (1.0 g, Aldrich Cat. 37, 763-5) by slurrying the absorbent in a solution of the extract in EtOH - H_2O (Ca 1 ml, mainly EtOH) and removing the solution under reduced pressure. This was then loaded onto a cotton wool pad at the top of a column of the C_{18}-silica gel (10 g, 8×2 cm) which had been dry-packed and then washed successively with CH_3CN, CH_3CN - H_2O (1 : 1), and H_2O (each ca. 20 ml). The column was developed as shown in the Fig. 1. An extract of *T. hatcheri* showed cytotoxic effects (P 388>150 μg / disk) against monkey kidney (BSC) cell lines and antifungal activity aganist the dermatophyte *Trichophyton mentagrophytes*. Reversed-phase flash chromatography concentrated the cytotoxic activity in fractions eluted with CH_3CN : H_2O, 3 : 1 and 9 : 1. This fraction contained one main UV-active compound by TLC. The unique absorption bands due to ester carbonyl band (1716 cm^{-1} and 1212 cm^{-1}) were shown in the IR spectrum along with hydroxyl group (3412 cm^{-1}) as well as aromatic group (1600, 1509 and 1436 cm^{-1}). The IR spectrum of compound showed the presence of conjugated carbonyl and aromatic group. Si-gel column chromatography spread cytotoxic activity across ethyl acetate - cyclohexane 3 : 97 and 5 : 95 fractions containing methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**) (Baek *et al.*, 1998). This compound was obtained pure in quantities, but too small for biological assays, synthesis and biological activity report here.

The least polar compound (**1**), purified by preparative TLC, had UV and IR spectra appropriate for a 3,4-dioxygenated benzoic acid derivative. The MS supported a molecular of $C_{18}H_{24}O_4$. The phenolic OH of **1** was observed in the 1H-NMR spectrum as a broad exchangeable signal at 5.7 ppm. The ^{13}C-

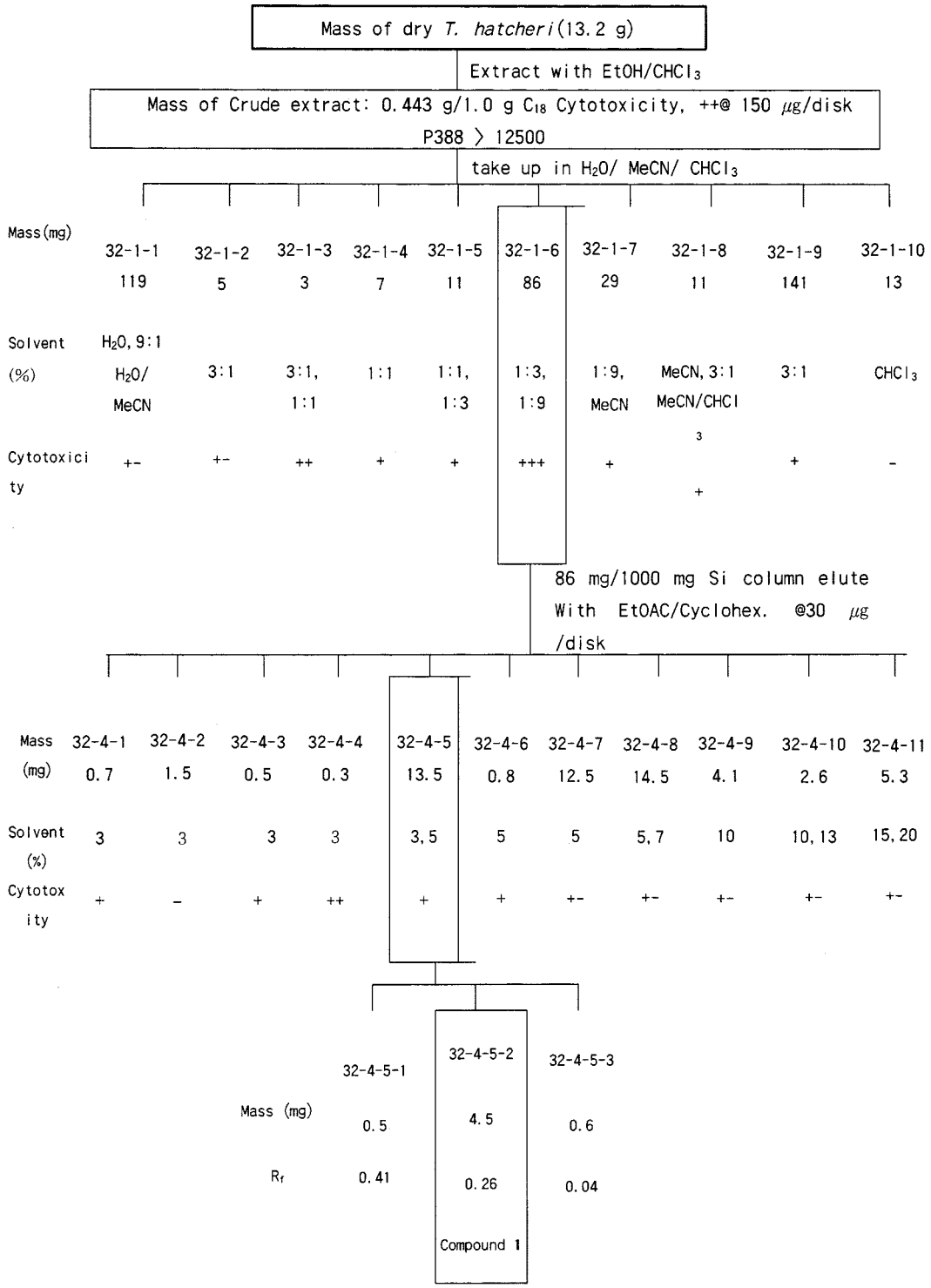

Fig. 1. Isolation of methyl-4-[{(2*E*)-3,7-dimethyl-2,6-octadienyl}oxy]-3-hydroxybenzoate (**1**) from *T. hatcheri*. [a]: Cytoxicity to BSC cells in 16 mm wells with a loading of 100 mg / 6 mm disk. +++: 4-6 mm zone. ++: 2-4 mm zone. +: 1-2 mm zone. + / -: minor effects located under disk.

NMR spectrum (Fig. 1) of our compound (**1**) showed signals appropriate for a trisubstituted aromatic ring, a geranyl group, an ester carbonyl, and methoxyl and hydroxyl groups, as expected for **1**. Because the ^{13}C-NMR spectra of **1** had not been reported, We rigorously assigned the ^{1}H and ^{13}C-NMR spectra

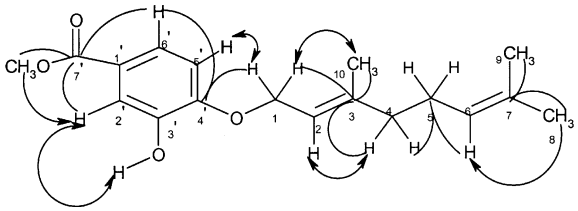

Fig. 2. Important NMR correlations establishing the structure of **1**.↔, selected NOE interactions; –, selected HMBC correlations.

with the aid of HMQC, HMBC, DEPT and NOE difference experiments. The NOE difference experiments produced a surprising result. Irradiation of the methoxyl and hydroxyl signals, which were barely resolved, only gave to enhancement of the H-2 aromatic proton signal. Structure **1** would be expected to give enhancements of both the H-2 and H-5. An NOE interaction between H-5 and H-1 protons of the geranyl group was also consistent with structure **1** (Fig. 2). The critical features that distinguish this structure are the linking of the geranyl group to the aromatic ring via an ether linkage instead of an ester and the replacement of a methyl ether with a methyl ester. Unequivocal evidence of these features was obtained from the HMBC experiment, in which correlations were observed between the ester carbonyl (δ166.8) and one hydroxyl proton signal (δ5.70) and between a quaternary oxygenated aromatic signal (δ149.7)

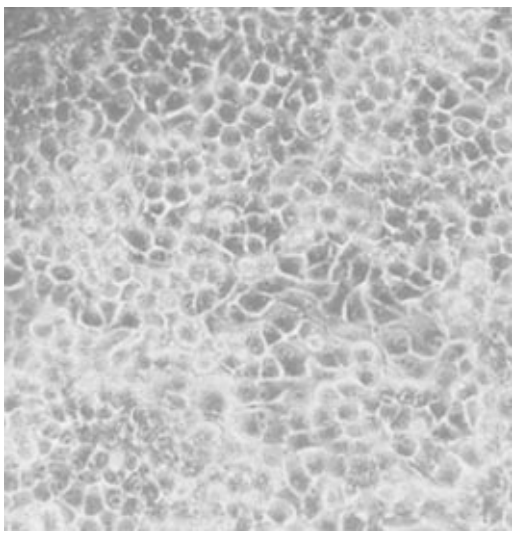

Photo 1. Inverted photomicrograph of KB cells after incubation in unmodified medium (control) for 2 days ×200. Most cells had abundant cytoplasmic process.

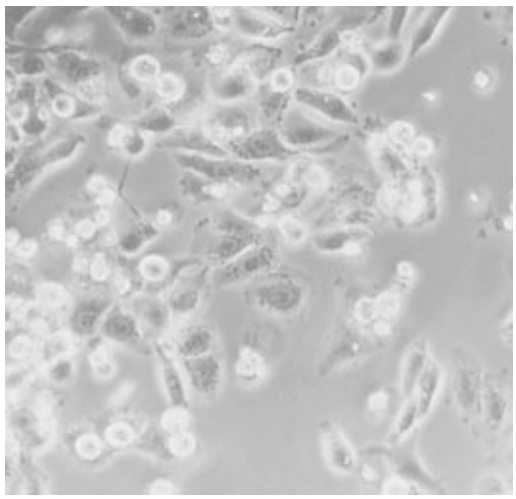

Photo 2. Inverted photomicrograph of KB cells after incubation in the medium containing 100 μM methyl-4-[{2*E*}-3,7-dimethyl-2,6-octadienyl}oxy]-3-hydroxybenzoate (1) for 2 days ×200. Most cells were formed cell cluster and number of cells were decreased.

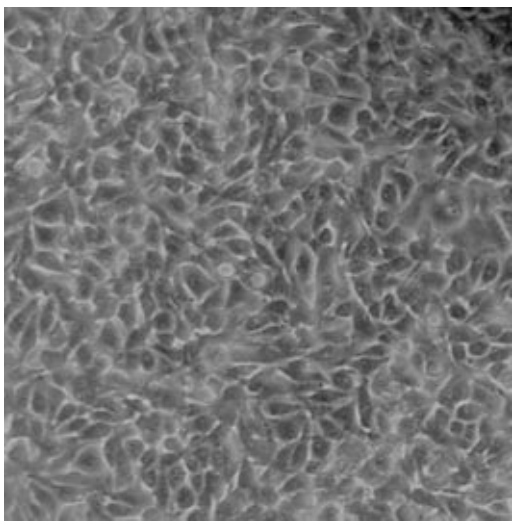

Photo 3. Inverted photomicrograph of SK-MEL-3 cells after incubation in unmodified medium (control) for 2 days ×200. Most cells had abundant cytoplasmic process.

and the H2-1 protons (δ4.67, d, J=7 Hz) of the geranyl group (Fig. 2) A further NOE interaction between the geranyl H-10 and H-1signals showed that the 2,3 double bond has *E* stereochemistery (Fig. 2).

A colorimetric assay was used to detect the *in vitro* antitumor activities mediated by methyl-4-[{(2*E*)-3,7-dimethyl-2,6-octadienyl}oxy]-3-hydroxybenzoate (**1**) against tumor cells. As shown in figures 1 and 3, this compound **1**-mediated antitumor activities

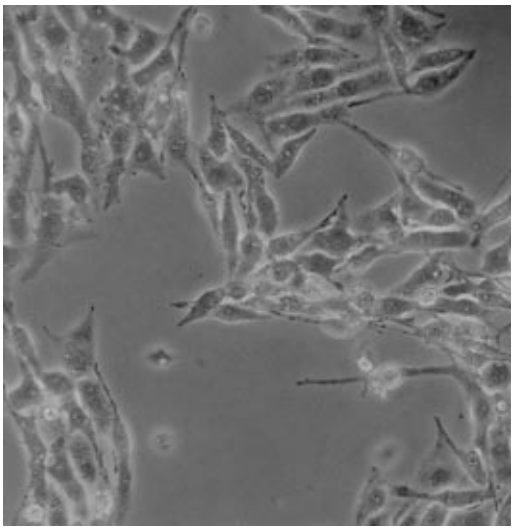

Photo 4. Inverted photomicrograph of SK-MEL-3 cells after incubation in the medium containing 100 μM methyl-4-[{2*E*}-3,7-dimethyl-2,6-octadienyl}oxy]-3-hydroxybenzoate (1) for 2 days ×200. Most cells were formed cell cluster and number of cells were decreased.

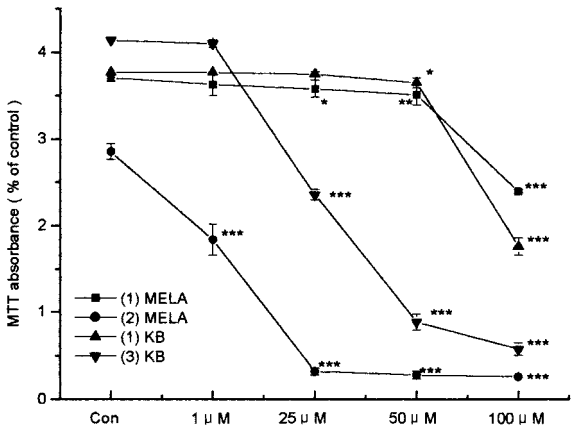

Fig. 3. *In vitro* antitumor activities of **1**, **2**, and **3** by the MTT assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of SK-MEL-3 and KB cell lines (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from four sets of experiments.

was not increased in the MTT assay when its concentration was raised from control 1 μM to 50 μM. However, its antitumor activities was rapidly increased in the MTT assay when its concentration was raised from 50 μM to 100 μM. 5-Fluorouracil as a reference was gradually increased in the MTT assay when its concentration was raised from 1 μM to 100 μM. This compound **1** and adriamycin as a reference against SK-MEL-3 cells was similar to KB

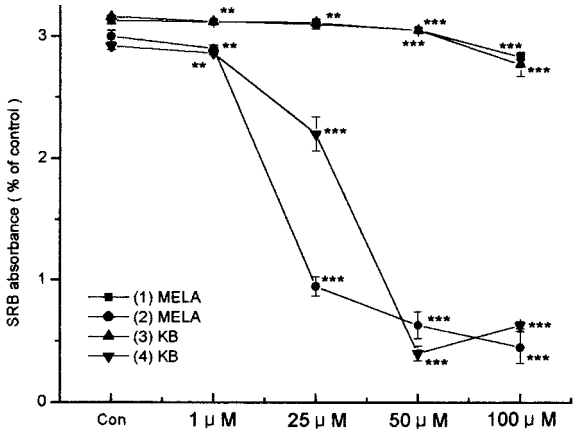

Fig. 4. *In vitro* antitumor activities of **1**, **2**, and **3** by the SRB assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of SK-MEL-3 and KB cell lines (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from four sets of experiments.

cells. As shown in Figures 2 and 4, this compound **1**-mediated antitumor activities was a little changeable in the SRB assay when its concentration was raised from control to 100 μM. However, its antitumor activities against SK-MEL-3 cells was similar to KB cells. Tables 1 and 2 show the potent antitumor activities of methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxy benzoate (**1**), 5-fluorouracil (**3**) and adriamycin (**2**) against KB cells and SK-MEL-3 cells. In general, the antitumor activities of these compounds were in a dose-dependent, and the susceptibility of the tumor cell lines to methyl-4-[(2*E*)-3,7-dimethyl-2,6-octadienyloxy]-3-hydroxybenzoate (**1**) was sensitive. The value of IC_{50} of **1**, **2** and **3** showed that compound **1** exerts the potent antitumor activity. The values of MTT_{50} and SRB_{50} on KB cells were determined at 113.71 μM

Table 1. The antitumor activities of **1**, and **2** on SK-MEL-3 cell lines

Compounds[a]	IC_{50} (μM)[b]	
	MTT assay	SRB assay
1	160.68	554.12
2	20.12	38.63

[a] Each compound was examined in four concentrations in triplicate experiments.

[b] IC_{50} represents the concentration of a compound required for 50% inhibition of cell growth.

Table 2. The antitumor activities of **1**, and **3** on KB cell lines

Compounds[a]	IC_{50} (μM)[b]	
	MTT assay	SRB assay
1	113.71	447.55
3	44.36	45.20

[a)]Each compound was examined in four concentrations in triplicate experiments.
[b)]IC_{50} represents the concentration of a compound required for 50% inhibition of cell growth.

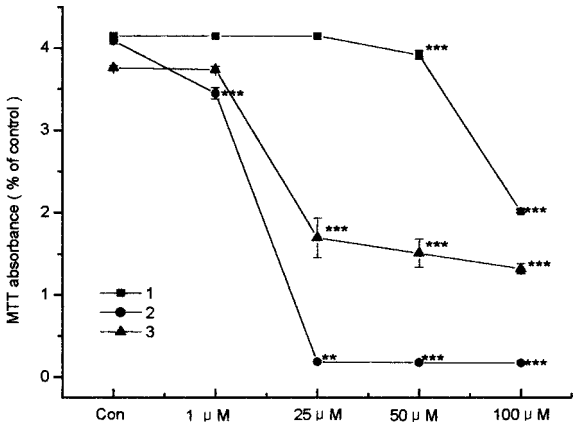

Fig. 5. *In vitro* cytoxicities of **1**, **2**, and **3** by the MTT assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from four sets of experiments.

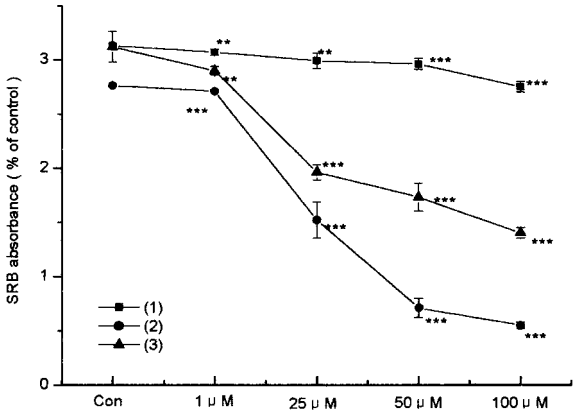

Fig. 6. *In vitro* cytoxicities of **1**, **2**, and **3** by the SRB assay. These compounds were serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of NIH 3T3 fibroblasts (5×10^4 cells). The colorimetric assay was performed as described in materials and methods. Data are mean values of results obtained from four sets of experiments.

Table 3. The cytotoxic effects of **1**, **2**, and **3** on NIH 3T3 fibroblasts

Compounds[a]	CD_{50} (μM)[b]	
	MTT assay	SRB assay
1	113.71	447.55
2	23.26	46.94
3	41.27	75.90

[a)]Each compound was examined in four concentrations in triplicate experiments.
[b)]IC_{50} represents the concentration of a compound required for 50% inhibition of cell growth.

and 447.55 μM, respectively (Table 1) and the values of MTT_{50} and SRB_{50} on SK-MEL-3 cells were determined at 160.68 M and 554.12 M, respectively (Table 2). References were evaluated for the strong antitumor activities against tumor cells. However. they have strong cytotoxic effects of NIH 3T3 fibroblasts (Oh *et al.*, 1999). A colorimetric assay was used to detect the *in vitro* cytotoxicity mediated by methyl-4-[{(2*E*)}-3,7-dimethyl-2,6- octadienyl}oxy]-3-hydroxybenzoate (**1**). As shown in Figure 5, this compound **1**-mediated cytotoxicity was rapidily increased in the MTT assay when its concentration was raised from 25 μM to 100 μM. However, this compound **1** was a little changeable in the MTT assay when its concentration was raised from control to 25 μM. The value of MTT_{50} was determined at 113.71 μM. As shown Figure 6, this compound 1-mediated cytotoxicity was a little changeable in the SRB assay when its concentration was raised from control to 100 μM. The value of SRB_{50} was determined at 447.55 μM. However, references **2** and **3** were rapidly increased in the MTT and SRB assays when their concentrations were raised from control to 100 μM.

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