JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antitumor Principle Constituents of *Myrica rubra* Var. *acuminata*

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Myrica rubra var. *acuminata* is a native shrub widely distributed and used as folk medicine in Taiwan for stomach disorders and diarrhea. Column chromatography combined with cytotoxic bioassay-guided fractionation was performed to isolate the antitumor principles from fresh leaves of *M. rubra* var. *acuminata.* The 20% MeOH eluate fraction of *M. rubra* var. *acuminata* inhibited the viability of HeLa and P-388 cells in an in vitro assay and an in vivo P-388 tumor-bearing CDF_1 mouse model. The percent increase in life span (%ILS) of 20% MeOH eluate fraction was greater than 125%. (−)-Epigallocatechin 3-*O*-gallate (**1**) and prodelphinidin A-2,3′-*O*-gallate (**2**) were isolated from D-20 as the antitumor principle components. Both compounds can inhibit the growth of HeLa cells, but **1** had lower cytotoxic effects in normal cervical fibroblasts than did **2**. Moreover, pretreatment with a caspase-3 specific inhibitor prevented **1**- and **2**-induced poly(ADP-ribose) polymerase cleavage. In view of these results, we suggest that **1** and **2** can induce apoptosis in HeLa cells and that activation of caspase-3 may provide a mechanistic explanation for their cytotoxic effects. Therefore, we suggest that the 20% MeOH eluate fraction extract is good for health and that *M. rubra* var. *acuminata* is an economically valuable plant.

KEYWORDS: ***Myrica rubra* var. *acuminata*; Myricaceae; apoptosis; HeLa cells; P-388 cells; (−)-epigallocatechin 3-*O*-gallate; prodelphinidin A-2,3′-*O*-gallate**

INTRODUCTION

Myrica rubra (Myricaceae) is widely distributed in Taiwan, and there is one variety, *M. rubra* Sieb. et Zucc var. *acuminata* Nakai (*1*). The bark of *M. rubra* has been used locally as an astringent, an antidote, and an antidiarrheic in Chinese traditional medicine (*2*). Previously, several flavonoids, tannins, triterpenes, and diarylheptanoids were isolated from the bark of *M. rubra* (*3*−*8*). Pharmacological studies of this medicinal plant have reported that its methanolic extract showed protective effects on CCl_4- and α-naphthylisothiocyanate-induced liver injury, whereas the 50% aqueous enthanolic extract and some constituents inhibited melanin biosynthesis and showed antiandrogenic activity (*9*). However, the constituents of *M. rubra* var. *acuminata* have not been investigated.

In our previous study, we screened the cytotoxicity effects of 70% acetone extracts of medicinal plants on HeLa cells using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay (*10*). The 70% acetone extract of *M. rubra* var. *acuminata* showed greater cytotoxic effects on cervical tumor cells than on normal cells. In the present study, cytotoxicity bioassay-guided fractionation was performed to isolate the antitumor principles from *M. rubra* var. *acuminata*, and their mechanism for inducing the cell death mode in tumor cells was investigated.

Several chemicals can induce tumor cell death; however, apoptosis is an efficient strategy for cancer chemotherapy. The apoptotic mode involves the active participation of affected cells in a self-destruction cascade that culminates in DNA degradation via endonuclease activation, nuclear disintegration, and formation of "apoptotic bodies" that involve the cell remnants. These apoptotic bodies are rapidly cleaned from local tissues by macrophages (*11*). However, little is known about the regulation and induction of apoptosis by natural products. In the present study, the human cervical carcinoma cell line (HeLa) and mouse leukemia cell line (P-388) were differentially susceptible to apoptosis induced by a natural product. Cell death was detected and identified using an MTT assay, propidium iodide staining followed by flow cytometry, DNA electrophoresis, and poly-(ADP-ribose) polymerase (PARP) proteolysis by Western blot assay (*12*).

While many compounds have been shown to inhibit the proliferation of mammalian cells in culture, only a small proportion of these demonstrate significant selectivity in vivo even in the most chemosensitive animal tumor models. There-

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10.1021/jf026188i CCC: $25.00
Published on Web 04/12/2003

fore, the antitumor effects of the cytotoxic components were evaluated using a P-388 tumor-bearing CDF_1 mouse model (*13*).

MATERIALS AND METHODS

General. ^1H (500 MHz) and ^{13}C NMR (126 MHz) spectra were measured on a Bruker DRX 500 instrument, and chemical shifts are given in δ (ppm) values. Values of fast atom bombardment mass spectrometry (FAB-MS) were obtained on a VG 70-SE mass spectrometer using 3-nitrobenyl alcohol containing NaCl as the matrix agent. Normal phase high-performance liquid chromatography (HPLC) was conducted on a 250 mm × 4 mm i.d. LiChrospher Si60 column (Merck, Darmstadt, Germany) using *n*-hexane−MeOH−tetrahydrofuran (THF)−HCOOH (47:42:10:1) containing 450 mg/L oxalic acid as the mobile phase. The flow rate was 1.0 mL/min, with detection at 280 nm. Reversed-phase HPLC was conducted on a 250 mm × 4 mm i.d. LiChrospher 100 RP-18e column (Merck) eluted with 0.05 M H_3PO_4−0.05 M KH_2PO_4−CH_3CN (44:44:12). The flow rate was 1.0 mL/min, and detection was at 280 nm. Column chromatography was carried out on Toyopearl HW-40 (coarse grade) (Tosoh), Diaion HP-20, and MCI-gel CHP-20P (Mitsubishi Chemical Industry, Japan). All solvents used for column chromatography were of analytical grade.

Dimethyl sulfoxide (DMSO), MTT, trypan blue, and other chemicals were purchased from Sigma Industries (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics, glutamine, and trypsin−ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY). Western blotting was performed using an antibody specific to human PARP (sc-7150), α-tubulin (sc-8035), antirabbit IgG-AP (sc-2007), and antimouse IgG-AP (sc-2008), which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents and chemicals were of the highest purity grade available.

Plant Materials. Fresh leaves of *M. rubra* var. *acuminata* were collected in Taipei, Taiwan, in September 2000 and dried at below 40 °C to yield 5.0 kg of dried leaves. A voucher specimen (MR001) is deposited in the Graduate Institute of Pharmacognosy Science, Taipei Medical University.

Isolation. Dried leaves (5.0 kg) were homogenized in 70% aqueous acetone (50 L) and filtered. The concentrated filtrate (574 g) was chromatographed over a 45 cm × 9.5 cm i.d. Diaion HP-20 column (Mitsubishi Chemical Industry) with aqueous MeOH (10% → 20% → 40% → 60% MeOH) and acetone. The 20% MeOH eluate (51.39 g) was rechromatographed over an ODS column eluted with 0.05 M KH_2PO_4−0.05 M H_3PO_4−CH_3CN (44: 44: 12) and MCI-gel CHP20P with aqueous MeOH to yield (−)-epigallocatechin gallate (**1**, 181.0 mg) and prodelphinidin A-2,3′-*O*-gallate (**2**, 63.2 mg) (**Figure 1**). Each compound was identified by direct comparison of its spectroscopic data with authentic samples (*4*, *5*, *14*). Purity tests of the two compounds were shown to be greater than 95% by normal and reversed phase HPLC. Retention times for normal and reversed phase HPLC were 11.2 and 12.9 min for **1** and 15.3 and 22.8 min for **2**, respectively.

(−)-Epigallocatechin Gallate (1). Pale yellow amorphous powder. FAB-MS: *m*/*z* 459 $[M + H]^+$. ^1H NMR (500 MHz, acetone-d_6): δ 2.89 (1H, dd, $J = 2.3$, 17.3 Hz, H-4β), 3.02 (1H, dd, $J = 4.5$, 17.3 Hz, H-4α), 5.05 (1H, s, H-2), 5.54 (1H, br t, $J = 2.3$ Hz, H-3), 6.01 (1H, d, $J = 2.4$ Hz, H-6), 6.04 (1H, d, $J = 2.4$ Hz, H-8), 6.61 (2H, s, H-2′,H-6′), 7.01 (2H, s, Gal H-2,H-6). ^{13}C NMR (126 MHz, acetone-d_6): δ 78.1 (C-2), 69.2 (C-3), 26.6 (C-4), 157.1 (C-5), 95.8 (C-6), 157.8 (C-7), 96.5 (C-8), 157.4 (C-8a), 99.0 (C-4a), 130.7 (C-1′), 106.7 (C-2′, C-6′), 146.2 (C-3′, C-5′), 133.1 (C-4′), 121.9 (Gal C-1), 110.0 (Gal C-2, C-6), 145.9 (Gal C-3,C-5), 138.8 (Gal C-4), 166.0 (ester carbonyl).

Prodelphinidin A-2,3′-*O*-gallate (2). Tan amorphous powder; $[\alpha]_D$ −132° ($c = 1.0$, acetone). FAB-MS: *m*/*z* 761 $[M + H]^+$. ^1H NMR (500 MHz, acetone-d_6): δ 2.95 (1H, d, $J = 17.6$ Hz, H-4′β), 3.13 (1H, dd, $J = 4.9$, 17.6 Hz, H-4′α), 4.16 (1H, d, $J = 3.2$ Hz, H-3), 4.47 (1H, d, $J = 3.2$ Hz, H-4), 5.19 (1H, s, H-2′), 5.69 (1H, br d, $J = 4.9$ Hz, H-3′), 6.12 (1H, s, H-6′), 6.04 (1H, d, $J = 2.3$ Hz, H-6), 6.23 (1H, d, $J = 2.3$ Hz, H-8), 6.77 (2H, s, B-ring H-2, H-6), 7.12 (2H, s, Gal H-2, H-6), 6.78 (2H, s, B′-ring H-2, H-6). ^{13}C NMR (126 MHz, acetone-d_6): δ 104.0 (C-2), 68.3 (C-3), 27.5 (C-4), 151.4 (C-5), 96.2 (C-6), 152.4 (C-7), 97.7 (C-8), 99.9 (C-4a), 133.7 (B-ring C-1), 107.4 (B-ring C-2, C-6), 145.7 (B-ring C-3, C-5), 131.7 (B-ring C-4), 80.0 (C-2′), 67.9 (C-3′), 26.0 (C-4′), 155.8 (C-5′), 96.6 (C-6′), 157.9 (C-7′), 101.5 (C-4′a), 133.9 (B′-ring C-1), 107.1 (B′-ring C-2, C-6), 146.5 (B′-ring C-3, C-5), 129.3 (B′-ring C-4), 121.7 (Gal C-1), 110.5 (Gal C-2, C-6), 139.0 (Gal C-4), 145.8 (Gal C-3, C-5), 151.4, 153.8, 157.9 (C-5, C-7, C-8a), 152.4, 155.8, 156.5 (C-5′, C-7′, C-8′a), 166.3 (ester carbonyl).

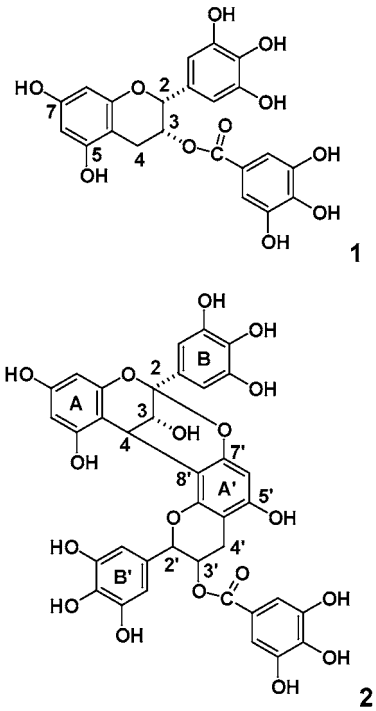

Figure 1. Structures of (−)-epigallocatechin 3-*O*-gallate, EGCG (**1**), and prodelphinidin A-2,3′-*O*-gallate, PAG (**2**).

Cell Cultures. The HeLa and P-388 cell lines were obtained from American Type Cell Culture (ATCC, Rockville, MD) and maintained in DMEM (Gibco) supplemented with 10% FBS, 100.0 mg/L streptomycin, and 100 IU/mL penicillin (Gibco). All cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Primary Culture. The normal cervical tissue was isolated from the cervical carcinoma in situ of a Taiwanese woman (45 years) and identified through the pathology by Dr. Chun-Sen Hsu in Wan-Fang Hospital, Taipei, Taiwan. Normal fibroblasts were isolated from human normal cervical tissues. Fresh tissues were rinsed twice with phosphate-buffered saline (PBS) and then cut into 0.1 cm^3 pieces. These pieces were incubated with 0.25% trypsin and 1.5 mg/mL type II collagease in DMEM for 4 h at 37 °C with 5% CO_2. Cells were then harvested in DMEM containing 10% FBS, 100 mg/L streptomycin, and 100 IU/mL penicillin, centrifuged for 10 min at 1200 rpm, and then seeded in culture dishes (*12*). After 1 day, the medium was changed to eliminate floating cells. Cells were used for experimental protocols from passages 1 to 3.

Cytotoxicity Assays. A stock solution of test samples (2×10^4 μg/mL) was prepared by dissolving tested samples in DMSO and then storing it at 4 °C until use. Serial dilutions of the stock solution were prepared in the culture medium in 96 well microtiter plates. Test samples at the appropriate concentrations were added to cell cultures for 48 h without renewal of the medium. The number of surviving cells was then counted using the tetrazolium (MTT) assay (*12*). The cytotoxicity index (CI%) was calculated according to the following equation: $CI\% = [1 - (T/C)] \times 100\%$, where T and C represent the mean optical density of the treated group and vehicle control group, respectively. In accordance with the CI% of the dose−response curve, the concentration of the test compound giving 50% of cell growth inhibition (IC_{50} value) was estimated.

Agarose Gel Electrophoresis. P-388 cells (1×10^6 cells/well) were treated with D-20 for 24 h, and the extent of DNA fragmentation was assessed by 1.5% agarose gel electrophoresis (*12*).

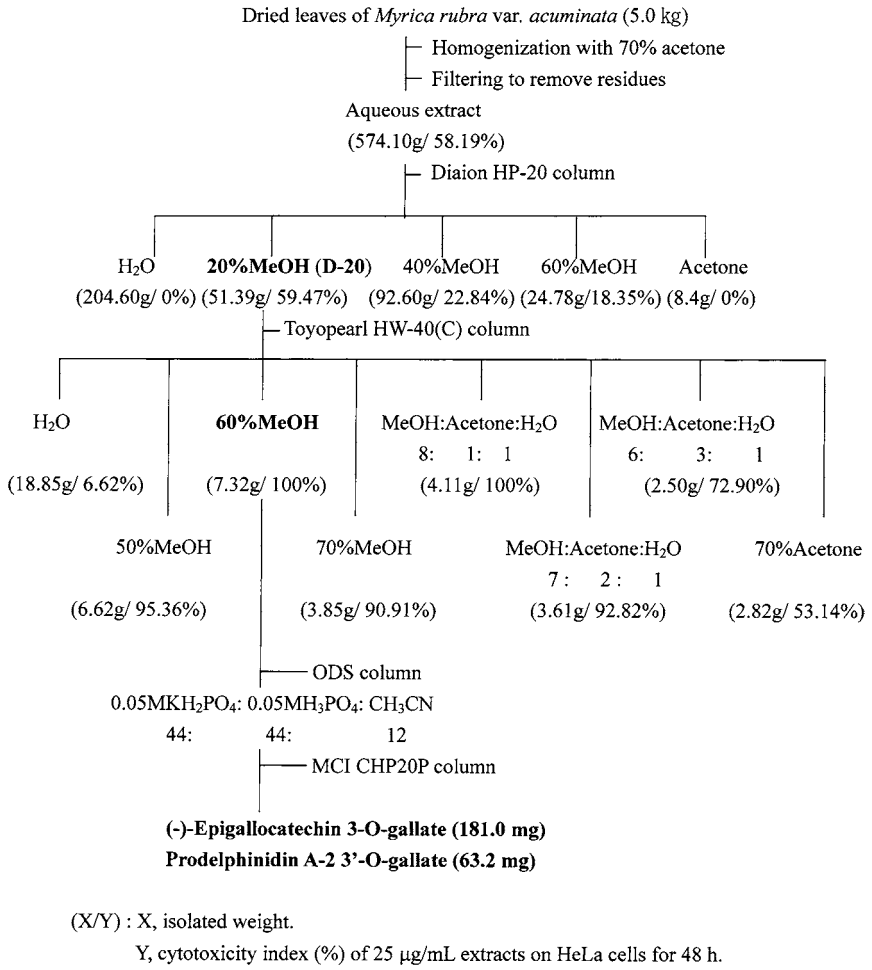

Figure 2. Isolation flowchart of *M. rubra* var. *acuminata* using bioassay-guided fractionation.

In Vivo Antitumor Assays. P-388 cells (1×10^6 cells/mouse) were transplanted intraperitoneally (ip) into 5 week old CDF_1 female mice (DBA male × BALB/c female) on day 0. D-20 was dissolved in normal saline. D-20 or normal saline was administered ip once a day on days 0−8. The antitumor effect was defined as the percent increase in life span (%ILS) calculated according to the following equation: $\%ILS = [(T - C)/C] \times 100\%$, where T and C represent the mean survival time (day) of the treated group and of the vehicle control group, respectively. The body weight of each CDF_1 mouse was determined every day using an animal scale. Data are presented as the mean ± standard deviation. Student's *t*-test was used for comparison of body weight and survival time (day) between the test and the blank groups (*13*).

Flow Cytometry Analysis. After appropriate treatment, HeLa cells (5×10^5 cells/well) were harvested by centrifugation and washed with PBS. Cells were fixed in ice-cold 80% ethanol, treated with 1.0 mg/mL RNase A, and stained with 50 μg/mL propidium iodide. Samples were run through a FACScan (Becton Dickinson, San Jose, CA). Results are presented as the number of cells vs the amount of DNA as indicated by the intensity of fluorescence (*12*).

Western Blot Analysis. HeLa cells (5×10^5 cells/well) exposed to **1** or **2** for 48 h were collected into tubes and then washed with PBS. Cell pellets were lysed with lysis buffer containing 40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonide P-40, and protease inhibitors. Total proteins (50 μg) were used for Western blot analysis. Western blot analysis was performed using 10% Tris−glycine−sodium dodecyl sulfate−polyacrylamide gels, and the protein was transferred to a nitrocellulose membrane by electroblotting. The membranes were probed with anti-PARP (a rabbit polyclonal antibody) and visualized using a BCIP/NBT kit (BCIP/NBT, Gibco) according to the manufacturer's instructions. As a loading control, we used anti-α-tubulin (a mouse monoclonal antibody) (*15*).

RESULTS AND DISCUSSION

The 70% aqueous acetone extract of *M. rubra* var. *acuminata* leaves was strongly cytotoxic to HeLa cells, with on IC_{50} of 21.69 μg/mL for 48 h. On the basis of the bioassay guide for fractionation, the aqueous extract was chromatographed on a Diaion HP-20 column to give five eluted fractions, and their cytotoxic effects are shown in **Figure 2**. Of the five fractions, fraction II (20% MeOH-eluted fraction, D-20) displayed the strongest cytotoxic effect. Therefore, the antitumor effects of D-20 were evaluated using murine P-388 leukemia in in vitro and in vivo models. In an in vitro assay, D-20 induced cell death and DNA fragmentation in a dose-dependent manner in P-388 cells with an IC_{50} of 24.8 μg/mL for 24 h (**Figure 3**). The effect of D-20 was evaluated for its in vivo antitumor activity against intraperitoneally implanted P-388 leukemia in CDF_1 mice. D-20 at 18.75 mg/kg body weight could prolong the life span of P-388 tumor-bearing CDF_1 mice by more than 125% as compared to normal saline-treated mice (**Table 1**). However, the high doses of D-20 (37.5 and 75.0 mg/kg) caused a toxic reaction in these mice, D-20-treated group, such that the ILS% was below 100% (**Table 1**). The P-388-bearing mice were continuously injected with D-20 for 9 days, and the body weights of high dose groups quickly decreased in this period. This situation may be due to high doses of D-20, which could kill tumor and normal immune cells and decrease the immune functions. When the P-388-bearing mice stopped treatment with high doses of D-20, the tumor cells recurred more quickly than normal cells and body weights significantly increased. In the 18.75 mg/kg D-20-treated group, the mean body weight of the mice was significantly lower

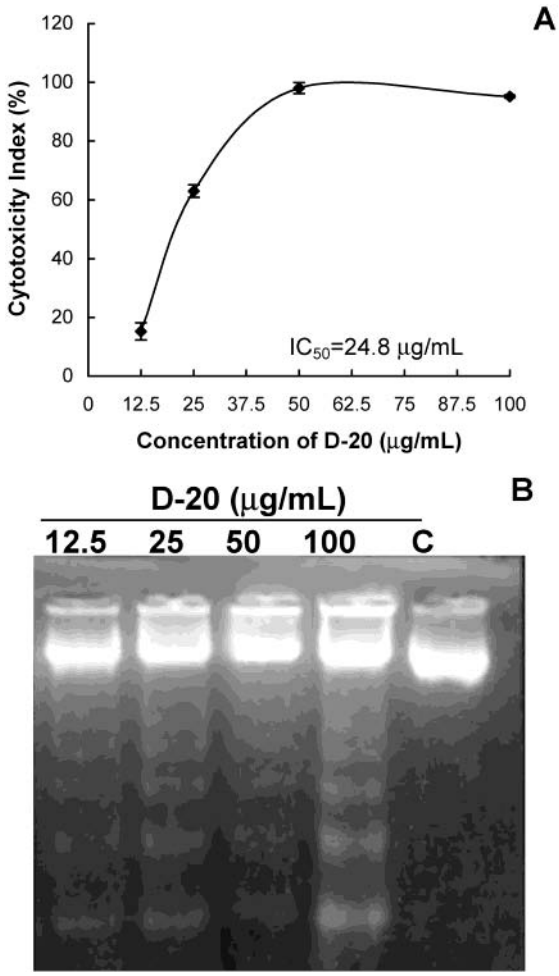

Figure 3. D-20 extract-induced cytotoxicity and DNA fragmentation in P-388 cells for 24 h. (**A**) Cytotoxic effects measured by MTT assay ($n = 3$). (**B**) DNA fragmentation detected by agarose gel electrophoresis. Data were used from three separate experiments, the picture for one of which is shown.

Table 1. Percentage Increase in Life Span (%ILS) of D-20-Treated P-388-Bearing CDF_1 Mice

group	survival time (day)	%ILS
blank (normal saline)	24.3	100.0
D-20 (mg/kg)		
18.75	≫60.0	≫125.0
37.50	22.5	92.8
75.00	17.5	72.2

than that of the control group on days 9−18 and ILS% could be prolonged (**Figure 4**). The suitable dose D-20 should kill tumor cells and damage the immune functions less. According to the above results, D-20 can inhibit the growth of P-388 cells in vitro and in vivo and is the major antitumor fraction extract of *M. rubra* var. *acuminata*. Therefore, D-20 was rechromatographed on a Toyopearl HW-40(C) column, and the cytotoxic effects of each developing fraction are shown in **Figure 1**. The higher yield and greater cytotoxic effect of the 60% MeOH-developed fraction were separated and purified using an ODS column to give **1** and **2**.

Compounds **1** and **2** were isolated from leaves of *M. rubra* var. *acuminata* as antitumor principles for the first time, and yields were 0.0036 and 0.0013%, respectively. The cytotoxic effects of these compounds exhibited dose-dependent effects at 5−40 μg/mL in HeLa cells for 24, 48, and 72 h (**Figure 5**).

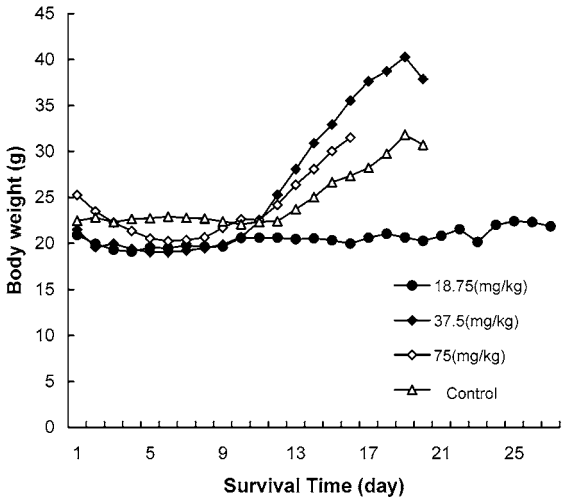

Figure 4. Effects of D-20 extract on body weights of P-388 tumor-bearing CDF_1 mice. The body weight of CDF_1 mice was evaluated every day while the mice were alive. Differences in body weights between the 18.75 mg/kg D-20 and the blank groups were significant on days 9–18 ($p < 0.05$, $n = 5$).

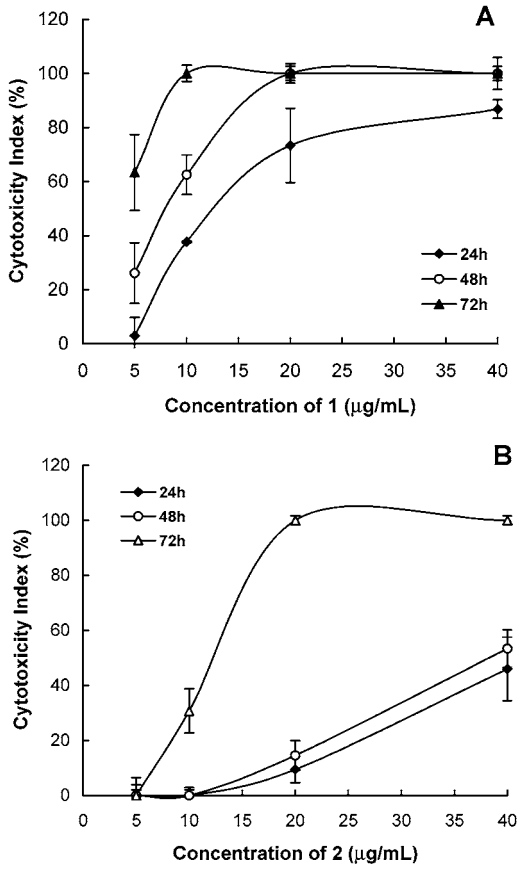

Figure 5. Concentration- and time-dependent cytotoxicity of **1** (**A**) and **2** (**B**) in HeLa cells by MTT assay ($n = 3$).

Compound **1** has fewer cytotoxic effects on primary normal cervical fibroblasts (NCFs) than on HeLa cells, but that was not the case for **2** (**Table 2**). Furthermore, the cytotoxic mechanisms of **1** and **2** were measured by fluorescence flow cytometry. DNA fragmentation is a characteristic feature of apoptosis (*16*). **Figure 6** shows that **1** and **2** induced DNA fragmentation at 10−80 μg/mL in HeLa cells for 48 h.

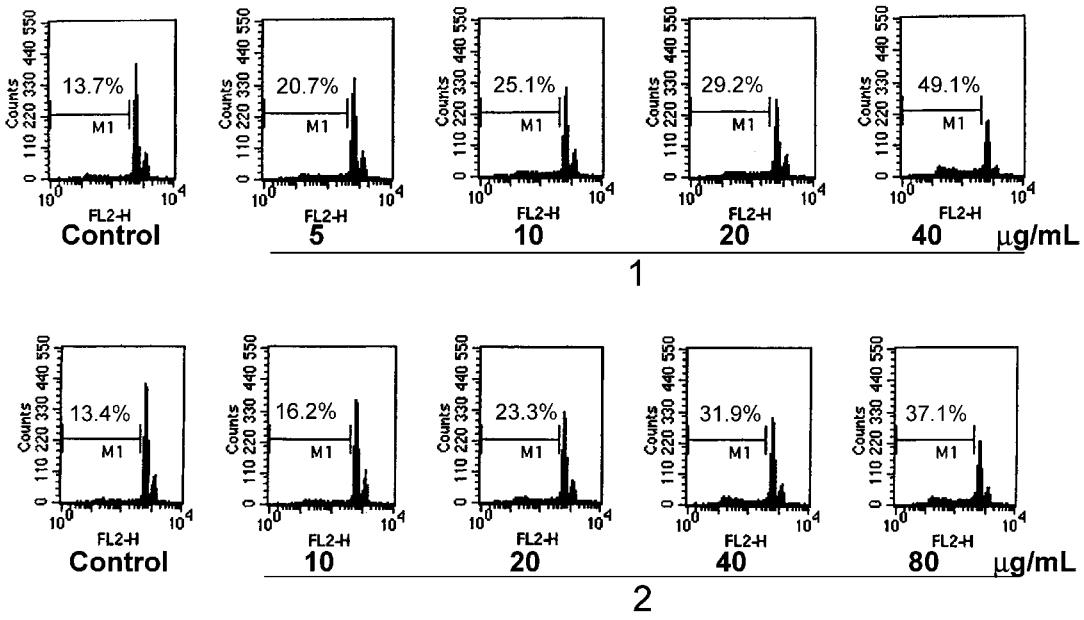

Figure 6. DNA content frequency histograms of HeLa cells after treatment with 10–80 μg/mL **1** (**A**) and **2** (**B**) for 48 h. Data were used from three separate experiments, one of whose picture is shown.

Table 2. IC_{50} Values of **1** and **2** on HeLa and Primary Culture Human NCFs after 48 h of Treatment

	IC_{50} (μg/mL)		
compd	HeLa	NCF	SI[a]
1	9.34	20.18	2.16
2	38.50	38.77	0.99
adriamycin	0.15	<0.15	<1.00

[a] SI, selectivity index; IC_{50} for NCF/IC_{50} for HeLa. Adriamycin is a positive control drug.

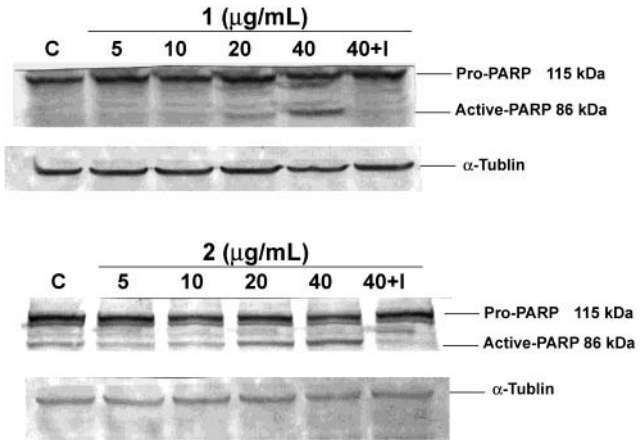

Figure 7. Western blot analysis of PARP and α-tubulin proteins in **1** (**A**)- and **2** (**B**)-treated HeLa cells for 48 h. α-Tubulin was used as an internal control to identify equal amounts of proteins loaded in each lane. C, 0.3% DMSO; 40 + I, HeLa cells pretreated with 100 μM caspase-3-specific inhibitor for 2 h, to which 40 μg/mL of the tested sample was added. Data were used from three separate experiments, the picture for one of which is shown.

Apoptosis produced the typical pattern of apoptotic PARP cleavage: a catalytically active band of intact PARP at 116 kDa and an active band at 85 kDa corresponding to the apoptotic cleavage product of PARP. PARP is proteolytically cleaved during apoptosis by caspase-3 (*17*), which reduces PARP's enzymatic activity (*18*), thereby inhibiting DNA repair. Treatment of HeLa cells with **1** and **2** stimulated proteolytic cleavage of PARP in a dose-dependent manner. Pretreatment with 100 μM of a caspase-3 specific inhibitor (Ac-Asp-Glu-Val-Asp-aldehyde) for 2 h inhibited **1**- and **2**-induced PARP cleavage (**Figure 7**). The above results suggest that **1** and **2** can induce apoptosis in HeLa cells and that activation of caspase-3 may provide a mechanistic explanation for their cytotoxicity effects.

Compound **1** is well-known as a chemopreventive agent, which is abundant in tea (*19*). In the present study, we now report that leaves of *M. rubra* var. *acuminata* contain **1** and that the D-20 extract can inhibit the growth of P-388 cells in vitro and in vivo. As previously reported (*9*), the 50% EtOH extract of leaves of *M. rubra* inhibited melanin biosynthesis in vitro and can possibly be used as a whitening agent for the skin. Therefore, we suggest that the D-20 fraction extract is good for health and that *M. rubra* var. *acuminata* is an economically valuable plant. In the future, **1** will be used as a biosubstance to control the quality of the D-20 fraction extract.

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Received for review December 3, 2002. Revised manuscript received March 6, 2003. Accepted March 7, 2003. We gratefully acknowledge the financial support (NSC90-2320-B-038-026) from the National Science Council, Republic of China.

JF026188I