Propolin G, a Prenylflavanone, Isolated from Taiwanese Propolis, Induces Caspase-Dependent Apoptosis in Brain Cancer Cells

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We have previously shown that six propolins, A–F, could be isolated from Taiwanese propolis (TP) and that they exerted a broad spectrum of biological activities. Recently, we isolated a seventh compound, propolin G. Its chemical structure has been identified by NMR and fast atom bombardment–mass spectrometry spectra and was found to be identical to a known compound, nymphaeol C. We used high-performance liquid chromatography to determine the relative contents of propolins C, D, F, and G in TP collected in various seasons and regions and found them to be relatively higher in TPs collected from May to July than from September to October. In our present study, we were interested in the various biological activities of TP extract as well as in propolin G as a pure compound. We found that propolin G could efficiently induce apoptosis in brain cancer cell lines (glioma and glioblastoma). The apoptosis might have been through a mitochondrial- and caspase-dependent pathway. This result demonstrated that the TP collection season was more an important factor than the geographical region. Propolis has been suggested to possess a potent antioxidant activity. We further evaluated the antioxidant property of propolin G using DPPH (1,2-diphenyl-2-picryhydrazyl). Our results indicate that propolin G does possess free radical scavenging activity. We also evaluated the neuroprotective action of propolin G, TP, and BP (Brazilian propolis) extracts against oxidative stress in rat primary cortical neurons. Our data demonstrate that propolin G and TP extracts have a marked neuroprotective effect that is greater than BP extract. In conclusion, the isolation and characterization of propolin G from TP have demonstrated for the first time that this compound is a potent inducer of apoptosis in brain cancer cells and that this compound and TP extract exhibit a protective effect against oxidative stress in rat cortical neurons.

KEYWORDS: Taiwanese propolis; propolin G; cortical neurons; apoptosis; prenylflavanoid; brain cancer

INTRODUCTION

Propolis, a natural resinous product, is collected from various plant sources by honeybees, which use it to seal holes in their honeycombs. It is one of the world's most popular health food.

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In Taiwan, six propolins of the active components were isolated and characterized from Taiwanese propolis (TP) (*1–4*). Propolins have been shown to be powerful antioxidants with anticancer properties. Among the main classes of compounds found in TP are the prenylated derivatives of flavanone. They are present in rather high amount in TP and low amount in propolis from Okinawa, Japan (*5*). However, to our knowledge, these active ingredients have not been found in bee propolis from other countries or regions. TP is used widely in the traditional medicine of Taiwan and has been reported to possess a broad spectrum of biological activities, including anticancer (*3, 4*), antioxidant (*1*), and antimicrobial (*6–8*) activities.

Propolin G Isolated from Taiwanese Propolis

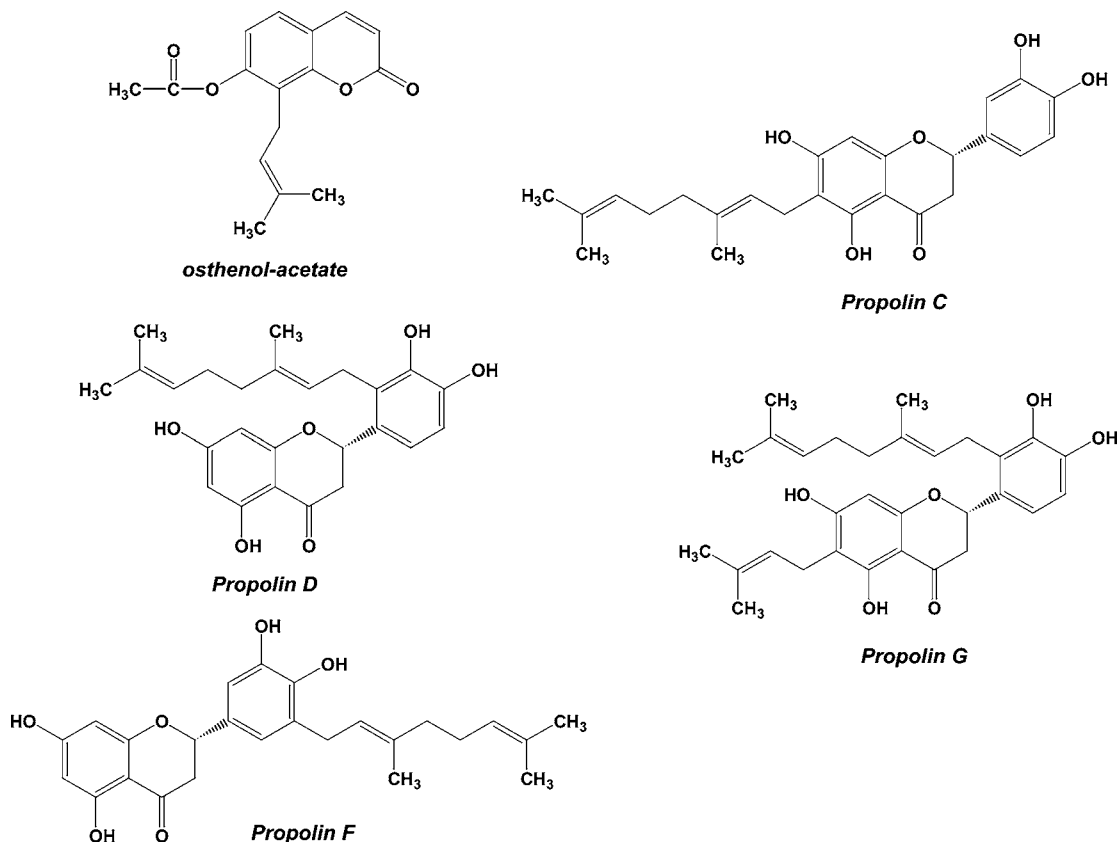*J. Agric. Food Chem.,* Vol. 55, No. 18, 2007   **7367**

Figure 1. Structures of the constituents identified from TP.

Propolis' chemical composition was complex and that this composition could vary by region or collection season. Recently, many reports have demonstrated that propolis from Europe and China contains high levels of flavonoids and phenolic acid esters (*9*). However, the major active components of another important propolis from Brazil contain terpenoids and prenylated derivatives of *p*-coumaric acids (*10, 11*). Several of these chemical compositions may be based on the botanical origins of propolis from different countries or regions.

In the present study, we have isolated a seventh active compound called propolin G. The chemical structure of propolin G has been identified by ^{1}H and ^{13}C NMR and fast atom bombardment—mass spectrometry (FAB-MS) spectra and was found to be identical to a well-known compound called nymphaeol C (*12*). As far as we know, no anticancer activities of this compound have ever been reported. We were interested in studying the activities that propolin G might have against brain cancer. Currently, 16000 people per year are diagnosed with brain cancer in the United States. Although it can occur at any age, many studies have indicated that it strikes most commonly in two age groups. The first group is children of 3−12 years old, and the second group is adults of 40−70 years old. The most frequent symptoms of brain cancer include headache, convulsion, nausea, loss of feelings in the arms or legs, and drowsiness. Recent treatment for brain cancer includes surgery, radiation therapy, and chemotherapy (*13*). However, the brain is a specific organ and controls the central nervous system. Any surgery or radiation on the brain produces side effects on the patient. A pressing need exists for the development of antibrain cancer therapies.

Apoptosis or programmed cell death has an essential role in controlling cell numbers in many developmental and physiological settings. Its morphological characteristics include cell shrinkage, nuclear condensation, plasma membrane blebbing,

chromosomal DNA fragmentation, and the formation of apoptotic bodies (*14*). Recent reports have shown that many anticancer drugs or cancer chemopreventive agents act through the induction of apoptosis to inhibit tumor promotion and progression. Mitochondria are currently regarded as playing a central role in mediating "intrinsic death signals" and could serve as a novel target for chemotherapies (*15, 16*). Cytochrome *c* is a mitochondrial protein that can activate caspase-9. However, the release of cytochrome *c* is regulated by the anti- and pro-apoptotic members of the Bcl-2 family (such as Bcl-2, Bcl-xL, Bax, Bak, Bad, and Bid). Once in the cytoplasm, it binds Apaf-1 to pro-caspase 9, leading to the activation of caspase-9 and triggering of the caspase cascade (*17*). Many parameters of mitochondrial physiology have been shown to be hallmarks of apoptosis. These include the loss of mitochondrial membrane potential ($\triangle \Psi m$), the generation of ROS (reactive oxygen species), the termination of oxygen consumption, and the release of cytochrome *c* (*18*). In the present report, we are focusing on the effect of propolin G on the apoptosis of brain cancer cells.

MATERIALS AND METHODS

Propolis Origins. TP was collected from two locations of Taiwan: Erhmei (in central Taiwan) and Fangliao (in southern Taiwan). The collectors, designed by Y. W. Chen and K. K. Ho, were developed so as to stimulate the bees to deposit more propolis of good quality in the collectors. The Brazilian propolis (BP, super green grade) was a gift from CONAP Ltd. (Brazil). Propolis from the collectors at each location was gathered every month and kept at −20 °C until processed. All samples were collected from May to December of 2000.

Extraction and Isolation of Propolin G. The TP (50 g) was extracted with 95% ethyl alcohol (250 mL × 3), sonicated for 3 h, and left to stand for 21 h at 25 °C. The filtered ethanol extract was evaporated to dryness under reduced pressure to yield a brown gum (34.5 g), which was kept at −20 °C until used. A portion of this extract
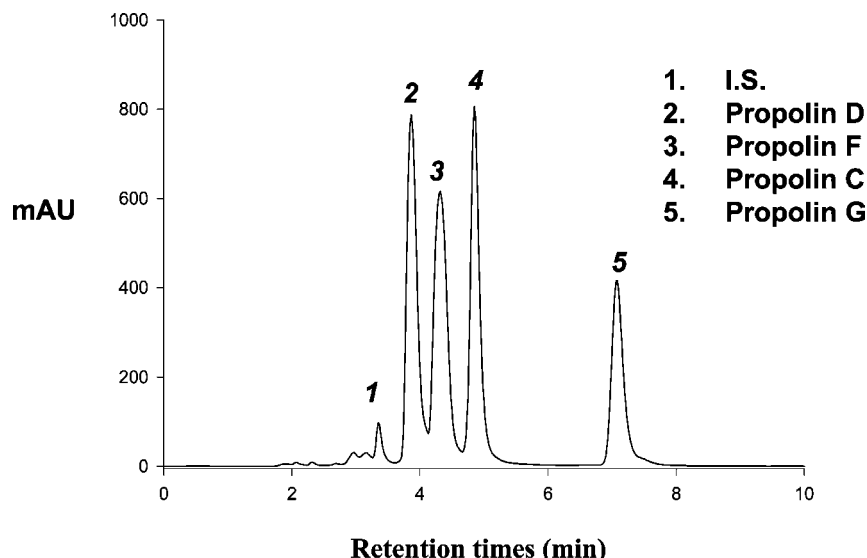
Figure 2. Separation of standard authentic compounds by HPLC/UV. Peaks: 1, osthenol-acetate (internal standard, 500 μg/mL); 2, propolin D; 3, propolin F; 4, propolin C; and 5, propolin G. The conditions of separation were as follows: column, Luna Phenomenex (C_{18}, 250 mm × 4.6 mm); mobile phase, methanol:water = 88.75, 11.25; flow rate, 1.0 mL/min; detection, UV 280 nm; and injection volume, 5 μL.

(5 g) was fractionated over a Sephadex LH-20 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using methanol as the solvent to elute and furnish six fractions. All eluates, including fractions from the follow-up chromatographies, were assayed on brain cancer cell growth inhibition, and the active fractions were again chromatographed on silica gel column using a gradient solvent system of *n*-hexane and EtOAc to elute. Purification of the most active fraction 3 (*n*-hexane: EtOAc, 70:30) was carried out on a reversed-phase (RP) preparative high-performance liquid chromatography (HPLC)/UV. Fractions of retention times at 25.0 min for propolin G were collected. Conditions were as follows: column, Luna Phenomenex (C_{18}, 250 mm × 10 mm); solvent system, methanol:water (8.5:1.5); flow rate, 3.5 mL/min; and detection, UV 280 nm. We identified the compound as propolin G (**Figure 1**). The purity of the propolin G was estimated to be no less than 95% by HPLC/UV based on the peak area.

Preparation of Samples and Propolins C, D, F, and G. TP extract samples were prepared at a concentration of 5 mg/mL. Previously, the major propolins C, D, and F (the chemical structures of which are depicted in **Figure 1**) contained in the TP extracts were isolated and characterized from our laboratory (*1*). Propolin C, D, F, and G powders were dissolved in methanol and prepared at a concentration of 1 mg/ mL. The purity of propolins C, D, and F was determined by HPLC/ UV. All propolin concentrations were determined using linear calibration curves based on the peak area for each propolin. Each calibration curve contained six concentrations of propolins: 1000, 500, 250, 125, 62.5, and 31.25 μg/mL.

Analysis Conditions. The propolin profile in the TP extracts was analyzed with a RP HPLC/UV. The conditions were as follows: column, Luna Phenomenex (C_{18}, 250 mm × 4.6 mm); solvent system, methanol:water = 88.75:11.25; flow rate, 1.0 mL/min; and detection, UV 280 nm.

Cell Culture and Cell Number Determination. Two cancer cell lines, rat C6 glioma and DBTRG-05MG (human glioblastoma) cells, were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Rat C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (FBS), a 1% dilution of penicillin−streptomycin, and 2 mM glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. DBTRG-05MG cells were cultured in RPMI 1640 (Gibco) with 2 mM glutamine and 0.1 mM NEAA, 100 mg/L sodium pyruvate, 10% FBS, and a 1% dilution of penicillin− streptomycin. All cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Propolin G was dissolved in dimethyl sulfoxide (DMSO) and prepared at a fixed concentration of 10 mg/mL. Cells (1 × 10^6 per dish) were cultured in a 100 mm dish and incubated for 14 h before being treated with DMSO or with various

concentrations of propolin G (2.5, 5.0, 7.5, and 10.0 μg/mL) for 24 or 48 h. Propolin G was a small compound (MW = 492). When these values were converted to molarities, they were 5, 10, 15, and 20 μM, respectively. Cells were counted and determined by a trypan blue exclusion assay.

Morphological Analysis of Apoptotic Cells. Brain cancer cells were cultured on six well culture plates and treated with propolin G at a concentration of 7.5 μg/mL for 24 h. After treatment, cells were fixed with methanol for 30 min, washed with phosphate-buffered saline (PBS), and then stained with 50 μg/mL of propidium iodide (PI) in the presence of 50 μg/mL RNase A. The morphology of nuclear chromatin was defined by the fluorescence of DNA-binding dye, PI, under a fluorescence microscope (Nikon, Japan).

Analysis of the Cell Cycle. Rat C6 glioma cells (1 × 10^6) in a 100 mm dish were treated with various concentrations of propolin G (0, 2.5, 5, and 10 μg/mL) for 72 h. Cells were trysinized and collected with ice-cold PBS. The cells were resuspended in 200 μL of PBS and fixed by adding 800 μL of iced 100% ethanol and then incubated overnight at −20 °C. The cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 1 μg/mL RNase A), and incubated at 37 °C for 30 min. Then, 1 mL of PI solution (50 μg/mL) was added, and the mixture was allowed to stand at 4 °C for 30 min. The cellular DNA content was then analyzed by FACScan cytometry (Becton Dickinson).

Activity of Caspase. Rat C6 glioma cells (1 × 10^6) in a 100 mm dish were treated with fixed concentrations of propolin G (12.5 μg/ mL) for 0, 4, 5, 6, and 7 h. Cells were collected and washed with PBS and suspended in a buffer containing 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM ethylenediaminetetraacetic acid, 5 mM dithiothione, 2 mM phenylmethylsulfonylfluoride, 10 μg/mL pepstatin A, and 10 μg/ mL leupeptin. After treatment, cell lysates were clarified by centrifugation at 13200g for 20 min at 4 °C. The caspase activity in the supernatant was determined by a fluorogenic assay (Caspase-3 Fluorometric Assay System, Sigma). Briefly, 100 μg of total protein, as determined by 7-amino-4-methylcoumarin (AMC) assay (Sigma), was incubated with 10 μM substrate DEVD-AMC at 37 °C for 1 h. The release of AMC was measured by excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (Hitachi F-4500).

Western Blotting Assay. Rat C6 glioma and DBTRG-05MG cells (1 × 10^6) in 100 mm dishes were treated with propolin G at various concentrations (0, 2.5, 5, 7.5, and 10 μg/mL) for 48 h. After treatment, cells were collected and resuspended in 100 μL of Gold lysis buffer. Equal amounts of proteins (30 μg) were mixed with 2× sample buffer and resolved by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis for β-actin, poly (ADP-ribose) polymerase (PARP), Bid, caspase-3, caspase-8, and caspase-9 detection. Proteins were elec-

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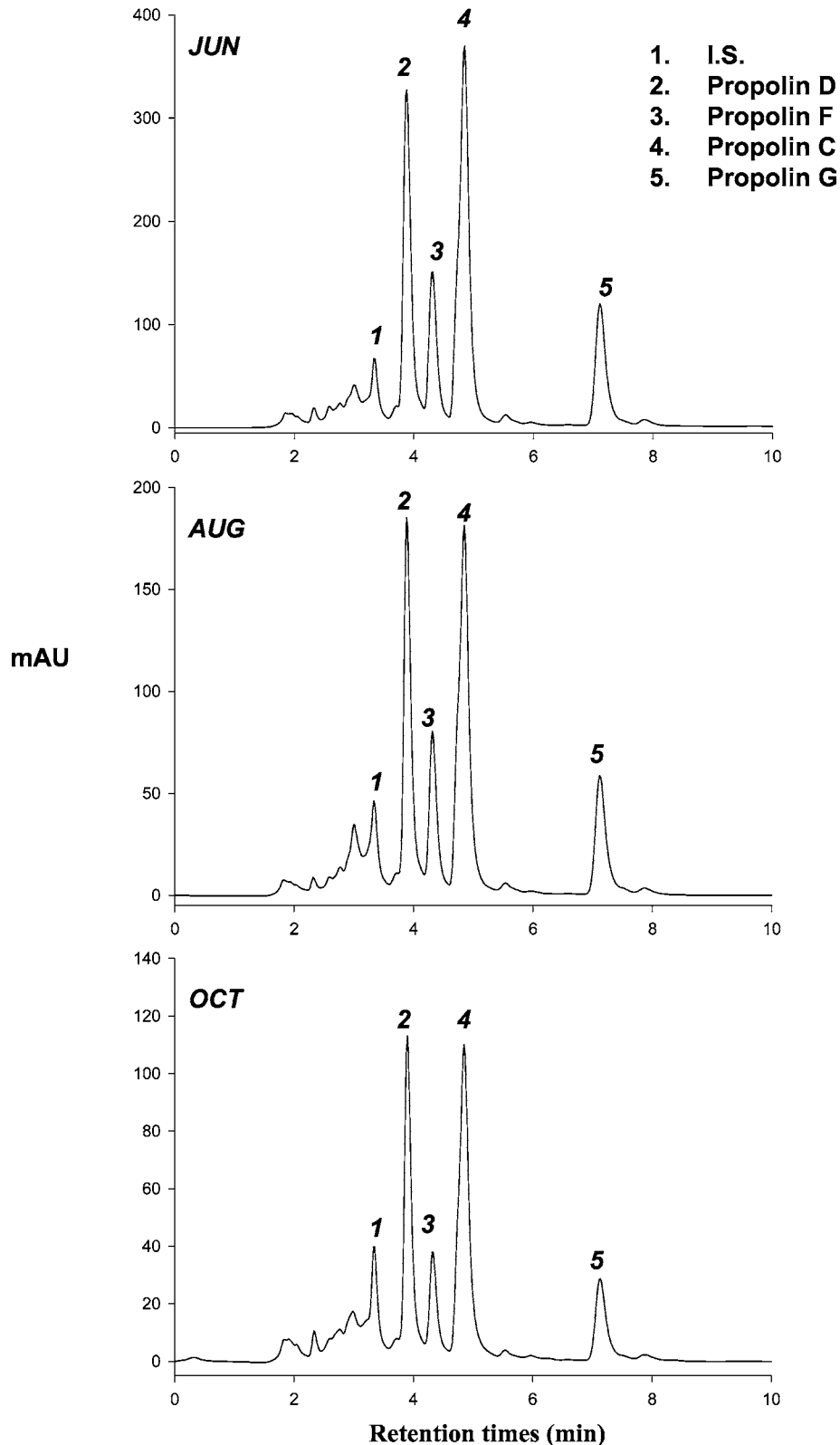*J. Agric. Food Chem.,* Vol. 55, No. 18, 2007 **7369**

Figure 3. Chromatography of different types of TP extracts of Fangliao:TW-I to TW-III of Fangliao TP. Peak identification and separation conditions are the same as those in **Figure 2**.

trotransferred to an immobilon membrane (PVDF; Millipore Corp.), and equivalent protein loading was verified by staining the membrane with reversible dye amido black (Sigma Chemical Co.). This was followed by overnight blocking with a solution composed of 20 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, and 3% BSA. Specific antibodies used were anti-human PARP and Bid (1:500 of rabbit polyclonal; Cell Signaling Technology, Inc.), anti-caspase-3, anti-caspase-8, and caspase-9 antibodies (1:800 of mouse monoclonal; Cell

Signaling Technology, Inc.). These proteins were detected by chemi-luminescence (Amersham).

Gene Expression Analysis. Total RNA from brain cancer cell lines was treated with propolin G (7.5 or 10 μg/mL) for 0, 1, 2, 4, and 6 h, or with various concentrations (0, 2.5, 5, 7.5, and 10 μg/mL) for 24 h, or with propolin G (7.5 μg/mL) for 24, 48, and 72 h. Cells were lysed, and the total RNA was extracted by a RNeasy Mini kit (Qiagen, CA). cDNA was prepared by a First Strand cDNA Synthesis Kit (Toyobo,

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Osaka, Japan). Multiplex polymerase chain reaction (PCR) was performed using the following primers for humans: p21[waf1] forward 5′-GGGGGGCATCATCAAAAACTT-3′, reverse 5′-ACTGAAGG-GAAAGGACAAGG-3′; cyclin B1 forward 5′-CGGGAAGTCACTG-GAAACAT-3′, reverse 5′-AAACATGGCAGTGACACCAA-3′; cyclin D1 forward 5′-CTGGCCATGAACTACCTGGA-3′, reverse 5′-GTCA-CACTTGATCACTCTGG-3′; cyclin E forward 5′-AATCGACAG-GACGGCGAGGGAC-3′, reverse 5′-GGCAGTCAACATCCAGGA-CACA-3′; glyceraldehydes-3-phosphate dehydrogenease (GAPDH) forward 5′-CCCATCACCATCTTCCAG-3′, reverse 5′-CAGTCT-TCTGGGTGGCAGT-3′ Multiple PCR was performed using the following primers for rats: p21[waf1] forward 5′-TGGCCTTGTCGCT-GTCTT-3′, reverse 5′-CTAAGGCAGAAGATGGGGAA-3′; cyclin B1 forward 5′-ACCAGAGGTGGAACTGGATG-3′, reverse 5′-TCTTCT-TGGGCACACAACTG-3′; cyclin D1 forward 5′-TGGAGCCCCT-GAAGAAGAG-3′, reverse 5′-AAGTGCGTTGTGCGGTAGC-3′; GAP-DH forward 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, reverse 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. After initial denaturation at 95 °C for 1 s, 30 cycles were performed at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s. The last cycle was followed by a 5 min extension at 72 °C.

2,2-Diphenyl-2-picryhydrazyl (DPPH) Free Radical Scavenging Activity. The free radical scavenging capacities of propolin G and CAPE (as a positive control) at various concentrations (2.5, 5, 10, 20, and 40 μM) were measured with 1.0 mL of 0.3 mM DPPH in methanol. The DPPH radical has a deep violet color due to its unpaired electron, and radical scavenging capability can be followed spectrophotometri-cally by an absorbance loss at 517 nm when the pale yellow nonradical form is produced. The mixtures were vigorously shaken and left to stand at room temperature for 30 min in the dark. Absorbance at 517 nm was measured vs methanol as a blank. The capability of scavenging DPPH radicals was then calculated by the following equation:

$$\text{scavenging effect} \% = [1 - (A_{517} \text{ of sample}/A_{517} \text{ of control})] \times 100\%$$

Primary Cortical Neurons. Primary cortical neuron cultures were prepared from 17 day old embryonic rat cerebral cortexes as originally described by di Porzio et al. (*19*) with minor modifications. In brief, the rat cerebral cortex was dissected and incubated with trypsin at room temperature for 5 min. Cells were then mechanically dissociated with a fire-narrowed Pasteur pipet in the culture medium and plated at a density of 600 cells/mm² in a six-well dish after counting the cell numbers with a trypan blue exclusion assay. Prior to use, all dishes were sequentially coated with 30 μg/mL poly-D-lysine and then with a neurobasal medium (Gibco) mixture containing B27 Supplement (Gibco). The culture medium contained 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Cells were cultured in the medium described above for 4 days in vitro (DIV). Cultures were maintained at 37 °C in a 5% CO$_2$/95% air-humidified incubator. After 4 DIV, cortical neurons were generated and pretreated with propolin G, TP, or BP extract for 2 h and then exposed to H$_2$O$_2$ (100 μM) in culture medium for 24 h, followed by determination of morphologic change using a microscope.

Densitometric Analyses for Gene Expression. Bands were scanned with BioImaging system (UVP, Inc., CA). In each case, bands were subjected to multiple exposures on the gel to ensure that the band density was in the linear range. The data are presented in terms of fold change over internal control (GAPDH) for each treatment. All data presented were from two to three independent experiments with similar results.

RESULTS AND DISCUSSION

Purification and Identification of Propolin G. The seventh active ingredient, called propolin G (**Figure 1**), was isolated through repeated chromatographies of the 95% ethanol extract of the propolis glue under the guidance of an anticancer activity test. Final purification of the active fraction was achieved by HPLC/UV on a RP column. The total content of the active component, propolin G, was roughly 3.0% of the propolis glue.

Table 1. Amount of Propolins C, D, F, and G in TPs from Different Seasons and Regions[a]

propolis source	group	μg/5 mg of propolis extract			
		propolin C	propolin D	propolin F	propolins G
		Fangliao			
June	TW-I	464.0 ± 13.1	305.0 ± 11.4	146.7 ± 7.1	237.9 ± 8.3
August	TW-II	315.1 ± 4.7	224.9 ± 12.1	102.8 ± 6.2	164.0 ± 7.1
October	TW-III	234.4 ± 12.0	166.0 ± 6.0	62.4 ± 3.1	101.5 ± 7.2
		Erhmei			
June	TW-II	779.6 ± 23.1	498.9 ± 17.3	221.4 ± 7.6	330.0 ± 11.8
July–August	TW-III	177.3 ± 6.4	107.4 ± 4.6	51.7 ± 3.8	78.1 ± 4.1
September	TW-III	ND	ND	ND	ND

[a] ND, not determined.

Both ^{1}H and ^{13}C NMR displayed absorption characteristics of the prenylflavanone type, very similar to those of propolin D; propolin D was more polar, isolated from the same source (*1*). The FAB-MS of a pure sample of propolin G indicated a [M]$^+$ of 492 with the molecular composition of C$_{30}$H$_{36}$O$_6$. Obviously, a short side chain of isoprene molecules was attached at the C6 site of ring A. Moreover, another long geranyl side chain is attached at the C2 site of ring B. A known compound, nymphaeol C, isolated from *Hernandia nymphaefolia*, happened to have the same side chain group at the C6 of ring A and C2 of ring B (*12*). Upon comparison of the ^{13}C NMR of propolin G in CD$_3$OD with the reported data of nymphaeol C, the chemical structure of propolin G could be recognized. Nym-phaeol C has been isolated from propolis collected in Okinawa, the southern-most prefecture of Japan, in the vicinity of Taiwan (*5*). Okinawa and Taiwan seem to have similar flora. Honeybees favored and collected these botanical sources of propolis, and Okinawan propolis has chemical compositions similar to TP. However, no anticancer activities of this compound (nymphaeol C) have ever been reported. In this study, we are interested in investigating the mechanism of inducing apoptosis in brain cancer cell lines.

Evaluation of Composition Change among Different Types of TP by HPLC/UV. A previous report of ours showed that the content of propolins A, B, and E in TP was low (*1*). In this report, we focus on the propolins contained in high amounts, C, D, F, and G. A mixture of four propolins, including C, D, F, and G, and the internal standard (osthenol-acetate was purified and characterized from our laboratory) was separated by HPLC/UV as described in the Materials and Methods, and a baseline resolution was achieved as shown in **Figure 2**. The separation of these TP constituents by HPLC/UV was completed in 10 min retention time. On the basis of color, we categorized TP into three types: TW-I (green), TW-II (brownish green), and TW-III (dark brown). These three different types of TP samples were analyzed. In general, TW-I was collected from May to June, TW-II was collected from July to August, and TW-III was collected from September to November from different areas of Taiwan. **Figure 3** shows the HPLC profile of TP extracts of Fangliao (located in southern Taiwan) from TW-I (June) to TW-III (October). It indicates that the TP extracts are rich in propolins C, D, F, and G and that concentrations of these compounds decrease significantly from June to October. A similar result shows more significant results in TP extracts of Erhmei (located in central Taiwan, data not shown). Further-more, we determined these two regional samples on the major active ingredients of propolins C, D, F, and G in different seasons and regions of Taiwan as shown in **Table 1**. HPLC/UV data support our color-based TP classification and also indicate that TP can be divided into three grades: first-grade
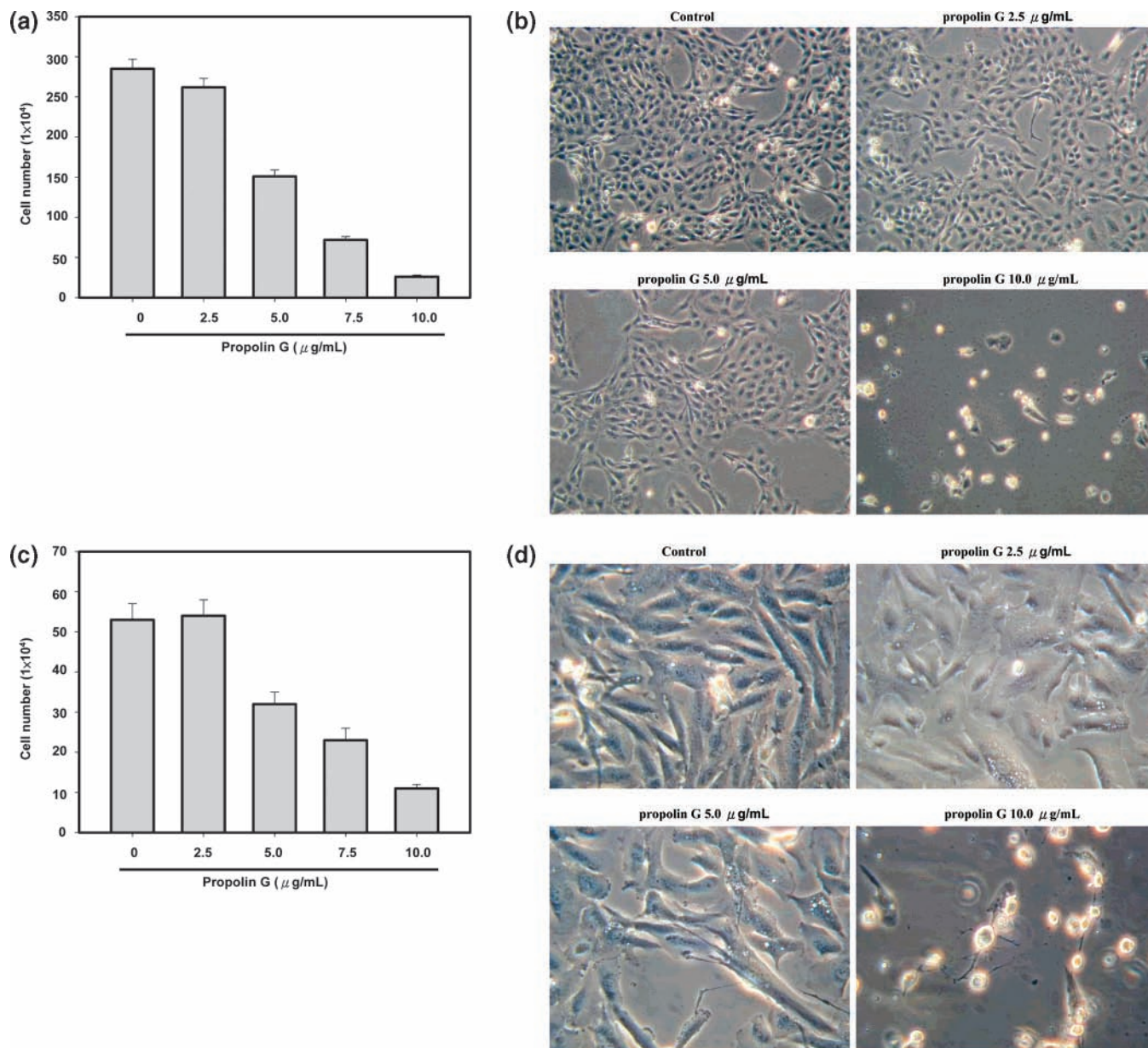
Figure 4. Effect of propolin G on the cytotoxic activity of two different types of brain cancer cell lines. (**a**) Cytotoxic activity of propolin G in various concentrations from 2.5 to 10.0 μg/mL for 48 h in rat C6 glioma cells or (**c**) in DBTRG-05MG cells. The treated cells were counted, and the cytotoxic activity was determined by a trypan blue exclusion assay. Results were from three repeats. (**b**) Phase-contrast micrographs of C6 glioma cells or (**d**) DBTRG-05MG cells treated with various concentrations of propolin G for 48 h. A representative experiment of three repeats is shown.

(TW-I), secondary-grade (TW-II), and third-grade (TW-III). Furthermore, we found that seasonality is more a key to determining the amount of propolins in TP than location; it may correlate with the amount of propolins contained in the botanical that change along with the seasonal changes. Next, we further evaluated the levels of propolins in botanical origin collected in different seasons. One of the TP source plants (Euphorbiaceae) was found. It was a popular tree in Taiwan, and the methanolic extracts of both its buds and young leaves were found to contain high levels of propolins (data not shown). Furthermore, young leaves of this plant of the same region were collected from summer to winter for evaluation. Our data indicated that the propolins, especially propolin G (data not shown), were low in the winter but high in the summer.

Inhibition of Cancer Cell Growth by Propolin G. The cytotoxic effects of propolin G on two brain cancer cell lines (rat C6 glioma and DBTRG-05MG) were investigated. These two cancer cell lines were treated with various concentrations

(2.5, 5, 7.5, and 10 μg/mL) of propolin G. After 48 h of treatment, the number of live cells was measured by a trypan blue exclusion assay. As shown in **Figure 4a,c**, both brain cancers cell lines were sensitive to propolin G, with an IC$_{50}$ of 5.0−7.5 μg/mL.

Propolin G Induces Apoptotic Cell Death in Brain Cancer Cells. Through a cell growth inhibition assay, we showed that propolin G induced significant cell death in two different brain cancer cell types. Inhibition of cell proliferation was seen in propolin G-treated cells. As shown in **Figure 4b,d**, the effects appear to be through the induction of apoptosis. The cytotoxic effect of propolin G was further shown to be evaluated as nuclear condensation under a fluorescence microscope as shown in **Figure 5a**. The morphological features of apoptosis, like condensation of chromatin and fragmentation of the nucleus, were seen in propolin G-treated cells. To investigate the
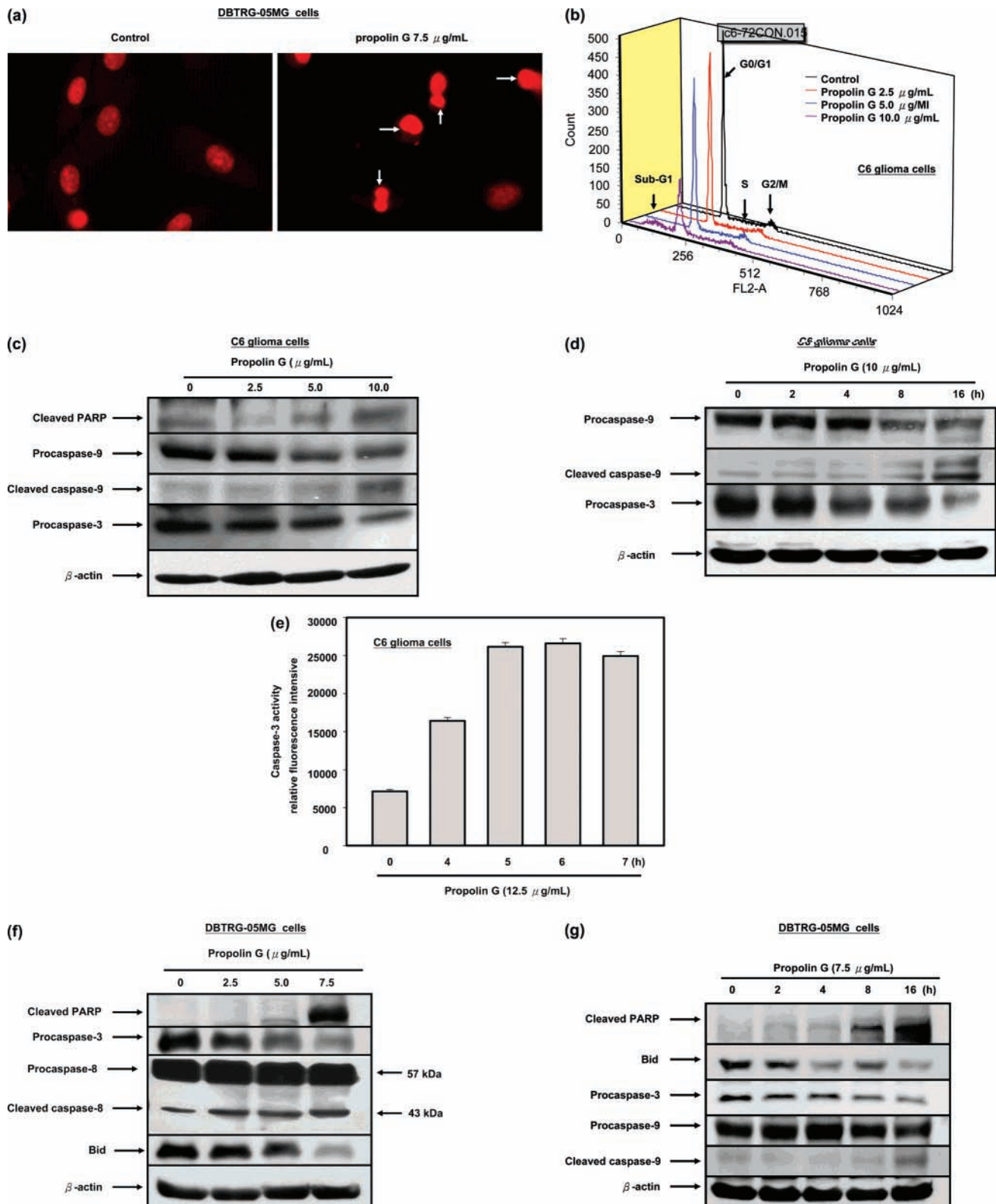
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Figure 5. Propolin G induces apoptosis in brain cancer cell lines. (**a**) Effect of propolin G on the morphology of nuclear chromatin. Untreated DBTRG-05MG cells and propolin G cells treated for 24 h were fixed and stained as described in the Materials and Methods. Morphological changes of nuclear chromatin were then viewed under a fluorescence microscope. (**b**) Flow cytometric analysis of propolin G-treated C6 glioma cells treated for 72 h and stained with PI as described in the Materials and Methods. Following flow cytometric analysis, the cellular DNA profile was analyzed by Cell Quest software. (**c**) Induction of apoptosis via caspase-dependent pathways such as caspase-9, caspase-3, and PARP. C6 glioma cells were treated with various concentrations of propolin G (2.5–10.0 μg/mL) for 48 h or (**d**) treated with propolin G (10.0 μg/mL) for 2, 4, 8, and 16 h. Western blotting assay as described in the Materials and Methods. (**e**) Activation of caspase-3 by propolin G. C6 glioma cells were treated with 12.5 μg/mL propolin G for 4, 5, 6, and 7 h, and after treatment, caspase-3 activities were measured. (**f**) Induction of apoptosis via mitochondria-dependent pathway. DBTRG-05MG cells were treated with various concentrations of propolin G (2.5–7.5 μg/mL) for 48 h or (**g**) treated with propolin G (7.5 μg/mL) for 2, 4, 8, and 16 h. Western blotting assay was as described in the Materials and Methods.

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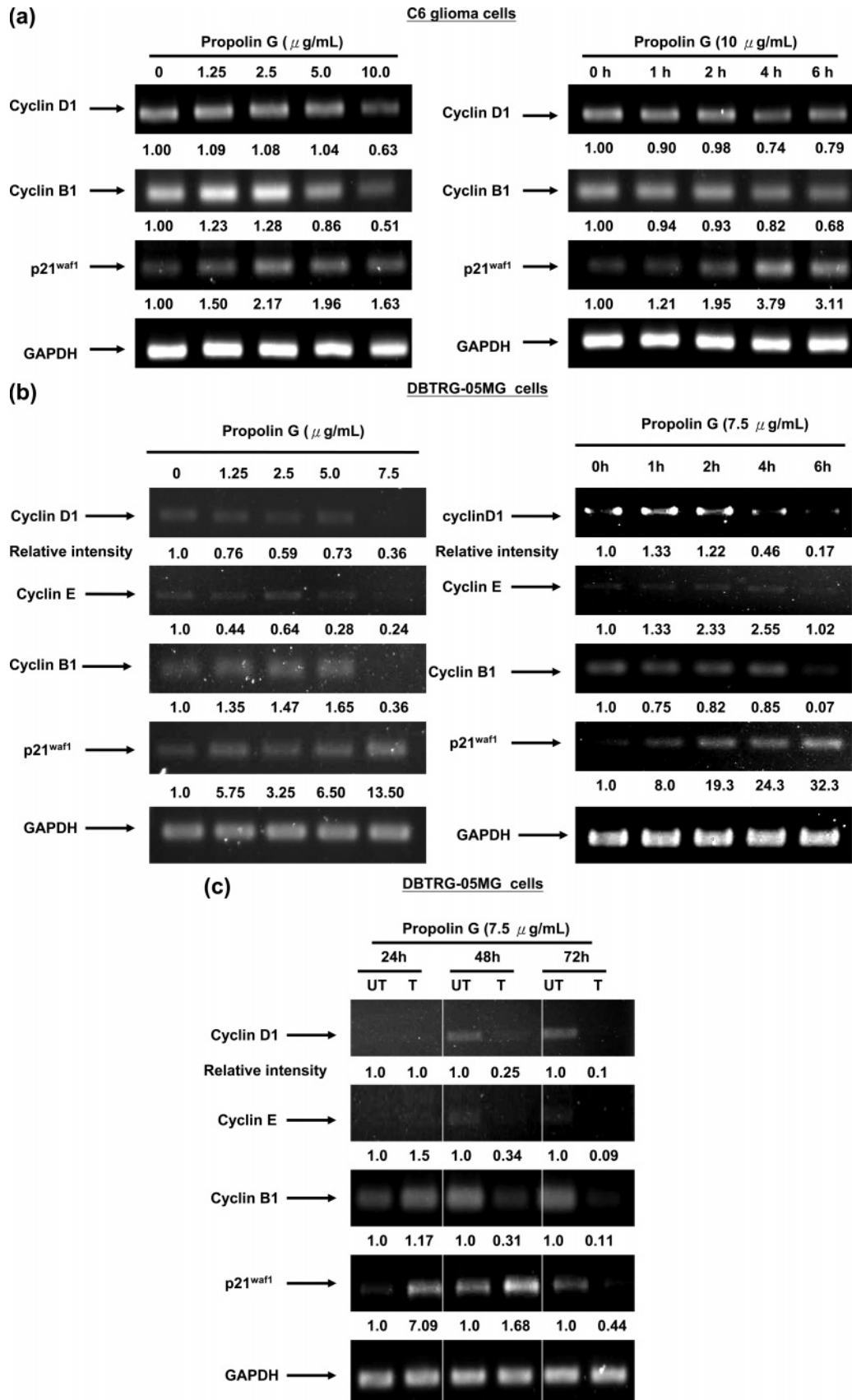*J. Agric. Food Chem.,* Vol. 55, No. 18, 2007   **7373**

Figure 6. Regulation of cell cycle regulators mRNA in propolin G-treated C6 glioma or DBTRG-05MG cells. (**a**) Rat C6 glioma cells were treated with indicated concentrations or treated with short times from 0 to 6 h. Cyclin B1, cyclin D, and p21 were determined by RT-PCR as described in the Materials and Methods. (**b**) DBTRG-05MG cells were treated with various concentrations of propolin G (2.5–7.5 μg/mL) for 24 h or treated with propolin G (7.5 μg/mL) for 1, 2, 4, and 6 h. Cyclin B1, cyclin D, cyclin E, and p21 were determined by RT-PCR as described in the Materials and Methods. (**c**) Long-term treatment with propolin G in DBTRG-05MG cells. Cells were treated with a fixed concentration of propolin G (7.5 μg/mL) for 24, 48, and 72 h. Cell cycle regulators mRNA were determined by RT-PCR as described in the Materials and Methods.
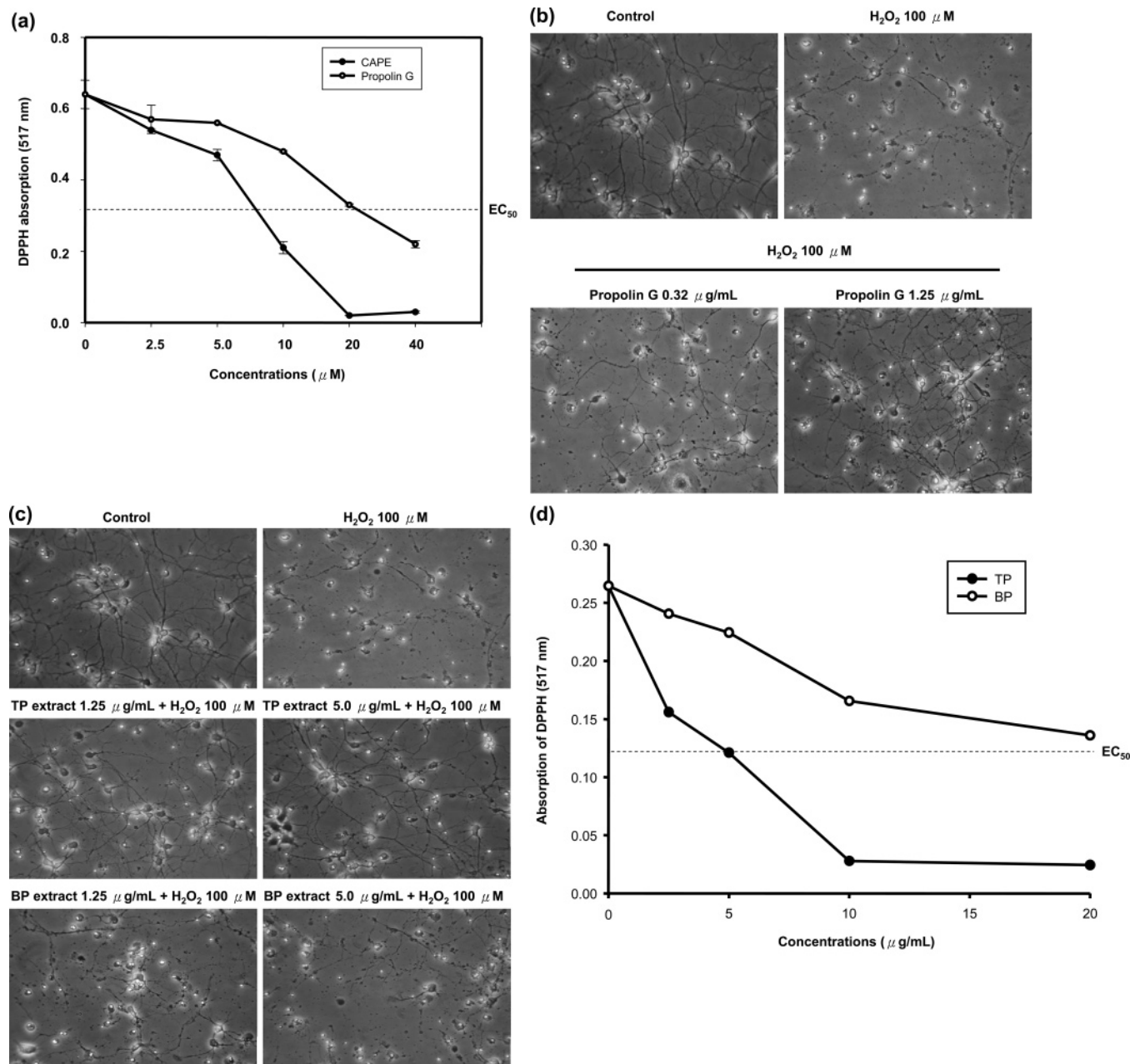
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Figure 7. Neuroprotection activity of propolin G and TP extract. (**a**) The DPPH-free radical scavenging activity of propolin G and CAPE was as described in the Materials and Methods. (**b**) Morphological observations. Phase-contrast micrographs of rat cortical neurons were cultured for 4 DIV and then pretreated with various concentrations of propolin G (0.32–1.25 μg/mL) for 2 h. Cells were exposed to H$_2$O$_2$ (100 μM) in culture medium for duration of 24 h. (**c**) The TP and BP extracts were evaluated under the same conditions. Cortical neurons for 4 DIV were pretreated with various concentrations of TP and BP extracts (1.25–5.0 μg/mL) for 2 h and then exposed to H$_2$O$_2$ (100 μM) for 24 h. (**d**) The DPPH-free radical scavenging activity of TP and BP extracts was determined as described in the Materials and Methods.

induction of a sub-G1 cell population, the DNA content of rat C6 glioma cells was treated with various concentrations of propolin G for 72 h and then analyzed by flow cytometry as shown in **Figure 5b**. The result indicated that propolin G markedly increased the sub-G1 cell population at a concentration of 10 μg/mL. All of these results demonstrated that propolin G efficiently induced apoptosis in two kind's brain cancer cells.

Propolin G Induces Apoptosis through Activation of the Caspase-Dependent Pathway. Apoptosis is a programmed cell suicide. It is triggered by many factors; caspases are the central components of this process. In mammalian cells, caspases involved in apoptotic effects are classified into two groups according to their function and structure. The first group is termed initiator caspases such as caspase-2, -8, -9, and -10,

which contain N-terminal adapter domains for autocleavage and activation of downstream caspases. The second group is termed effector caspases and includes caspase-3, -6, and -7, which lack N-terminal adapter domains and are cleaved and activated by initiator caspases (*20*). In this study, we focused on how propolin G induced apoptosis on the activation of caspase-3, caspase-8, and caspase-9 in two brain cancer cell lines. First, we evaluated whether caspase-dependent signal pathways were involved in the apoptotic effects induced by propolin G in brain cancer cells. Treatment of cells at various concentrations for 48 h or treatment with a fixed concentration (10 μg/mL) of propolin G for 0, 2, 4, 8, and 16 h resulted in a significant increase in the cleavage form of PARP (85 kDa) and in the active form of caspase-9 protein levels, and it decreased the procaspase-3 protein levels

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(**Figure 5c,d**). Activation of caspase-3 has been known to play an important role in the induction of apoptosis by various stimuli. Upon treatment of rat C6 glioma cells with 12.5 μg/mL propolin G, the activity of caspase-3 increased significantly within 4 h of treatment initiation as shown in **Figure 5e**. Because caspase-8 plays an important role both in the mitochondrial-dependent and -independent pathway in apoptosis (*21*), we further evaluated whether caspase-8 might be activated during the induction of apoptosis by propolin G, as shown in **Figure 5f,g**. Our results showed that propolin G markedly increased the active form of PARP and caspase-8 protein levels. All of these results indicate that PARP is markedly cleaved and caspase-8, caspase-9, and caspase-3 are markedly activated under treatment with propolin G.

Mitochondria-Dependent Pathway Was Activated during Propolin G-Induced Apoptosis. Cytosolic Bid is cleaved by caspase-8 and myristoylated before translation into mitochondria during the mitochondrial-dependent pathway of apoptosis (*22*). To determine if this pathway is activated by propolin G treatment, we next examined the Bid protein level after propolin G treatment with various concentrations for 48 h (**Figure 5f**) in brain cancer cells. The results indicate that Bid was markedly decreased after treatment with propolin G at a concentration of 5−7.5 μg/mL. In other evidence, we found that caspase-9 was activated by cytochrome *c* as shown in **Figure 5d**. Furthermore, the mitochondrial membrane depolarization was examined by using DiOC$_6$. The depolarization underwent marked change after treatment with propolin G (data not shown). Taken together, these results convincingly suggest that the propolin G-induced apoptosis of brain cancer cells may occur through the mitochondria-dependent pathway, as evidenced by the activation of caspase-8, Bid, caspase-9, and caspase-3.

Effect of Propolin G on Cell Cycle Regulators. Recently, study has demonstrated that cyclins D1 and E, and the cyclin-dependent kinase inhibitors p21 (Waf1/Cip1) are important regulators in cell cycle control and are called oncogenes or tumor suppressor genes (*23*). Many reports also demonstrated that cell growth inhibition may be regulated by cell cycle regulators, including the cyclin-dependent kinase inhibitor p21[waf1] (*24*), cyclin B1 (*25*), cyclin D1 (*26*), and cyclin E (*27*). We investigated the effect of propolin G on the inhibition of cell growth in brain cancer cell lines. Using semiquantitative RT-PCR, we studied these cell cycle regulators' gene expression following treatment with propolin G at various concentrations for 24 h or with a fixed concentration for different times. In each case, multiple gene analysis with GAPDH served to standardize mRNA levels over the course of the experiment. In rat C6 glioma cells, both cyclin B1 and cyclin D1 gene expression decreased markedly in a dose- or time-dependent manner. However, p21[waf1] increased markedly under propolin G treatment as shown in **Figure 6a**. Similar results were obtained in DBTRG-05MG cells as shown in **Figure 6b,c**. From these results, we conclude that inhibition of cell growth by propolin G may be via regulation of the expression of the cell cycle regulators, which may not necessarily implicate a direct causative induction of apoptosis. However, numerous studies have also revealed an association between cancers and cell cycle regulation, and the cell cycle regulators are also frequently found to be mutated in many human cancers (*28, 29*). Therefore, cell cycle regulation has been serving as a target for the control of cancers. In this study, we evaluated the anticancer activities of propolin G not only to determine the apoptotic mechanism but also to investigate its effects on the regulation of cell cycle regulators.

Free Radical Scavenging Activity. The free radical scavenging activities of the various concentrations of propolin G and CAPE [caffeic acid phenethyl ester, as a positive control (*30*)] were tested with DPPH, and the results are presented in **Figure 7a**. The EC$_{50}$ values of propolin G and CAPE were 20.5 and 8 μM, respectively. Propolin G seems to exhibit antioxidant activity; however, it is not a very strong antioxidant agent as compared with CAPE.

Neuroprotection by Propolin G and TP Extract. H$_2$O$_2$ is a stable, uncharged, and freely diffusible ROS (reactive oxygen species) with a second messenger in intracellular and extracellular signaling mechanisms. It can exert toxic effects by "Fenton reaction" (*31*), leading to the chemical structure change such as lipids, proteins, and DNA (*32*). In neurodegenerative situations such as Alzheimer's disease and Parkinson's disease, large amounts of H$_2$O$_2$ may be produced, which can be cytotoxic to neighboring cells (*33*). To further demonstrate the protective property of propolin G, cortical neurons of 4 DIV were exposed to H$_2$O$_2$ at 100 μM for 24 h in either the presence or the absence of propolin G at various concentrations. Cortical neurons were pretreated with various concentrations of propolin G (0.32−1.25 μg/mL) for 2 h (without changing the cell culture media) and then treated with H$_2$O$_2$ (100 μM) for 24 h (with propolin G and H$_2$O$_2$ copresent in the media). Our data demonstrated that propolin G at a concentration of 1.25 μg/mL significantly prevented the loss of cell viability induced by H$_2$O$_2$ as shown in **Figure 7b**. Next, similar treatment to confirm the protective property of propolis extract against cell death by oxidative stress in cultured cortical neurons was performed; two propolis (TP and BP) extracts were used. As shown in **Figure 7c**, cortical neurons were pretreated with various concentrations of TP or BP extract for 2 h and then exposed to H$_2$O$_2$ (100 μM) for 24 h. The TP extract exhibited significant neuroprotection at a concentration of 1.25−5.0 μg/mL. However, at the same concentrations of BP, the extract seems not to have any neuroprotective effect. Next, we also determined the free radical scavenging activity of TP and BP extracts. Our result showed that TP extract had stronger activity than BP extract as shown in **Figure 7d**. This result demonstrated that TP contains active ingredients (especially propolin C, data not shown), capable of protecting neurons from oxidative stress challenge. Recently, evidence has demonstrated that ROS may play an important factor in inducing neurodegenerative disorders, such as Alzhemier's, Parkinson's, and Huntington's diseases (*34, 35*). Dietary supplements that can scavenge ROS may serve to prevent or delay the progress of neurodegenerative diseases. Whether propolins can be detected in the plasma and pass through the blood−brain barrier after oral ingestion warrants further investigation. Propolis collected from the southeastern region of Brazil has been found to be rich in prenylated phenylpropanoids. Prenylated cinnamic acids, such as artepillin C, were major ingredients contained in Brazilian green propolis (*36*). Brazilian green propolis is derived mainly from vegetative apices of *Baccharis dracunculifolia* (alecrim plants). We conclude that active ingredients contained in BP are very different from those in TP.

In summary, we think that propolin G-induced growth inhibition and apoptosis of brain cancer cell lines may result from modulation of cell cycle regulators' gene expression and further activation of caspases and mitochondria pathways, finally resulting in the induction of apoptosis in brain cancer cell lines. On the other hand, we also showed that the compositions (propolins C, D, F, and G) change among TPs collected from different seasons. Finally, we indicated that propolin G and TP

extract exhibit the prevention of neuronal death against oxidative stress challenges in rat cortical neurons.

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