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Antiproliferation Effect and Apoptosis Mechanism of Prostate Cancer Cell PC-3 by Flavonoids and Saponins Prepared from *Gynostemma pentaphyllum*

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ABSTRACT: The objectives of this study were to investigate the antiproliferation and apoptosis mechanism of saponin and flavonoid fractions from *Gynostemma pentaphyllum* (Thunb.) Makino on prostate cancer cell PC-3. Both flavonoid and saponin fractions were isolated by a column chromatographic method with Cosmosil 75C_{18}−OPN as adsorbent and elution solvents of ethanol−water (30:70, v/v) for the former and 100% ethanol for the latter, followed by high-performance liquid chromatography−tandem mass spectrometry analysis. On the basis of the MTT assay, the saponin and flavonoid fraction were comparably effective in inhibiting the growth of PC-3 cells, with the IC_{50} being 39.3 and 33.3 μg/mL, respectively. Additionally, both fractions induced an arrest of PC-3 cell cycle at both S and G2/M phases, with both early and late apoptotic cell populations showing a dose-dependent rise. The Western blot assay indicated that the incorporation of flavonoid or saponin fraction could modulate the expression of G2 and M checkpoint regulators, cyclins A and B, and the antiapoptotic proteins Bcl-2 and Bcl-xl and pro-apoptotic proteins Bad and Bax. The expression of the caspase-3 and its activated downstream substrate effectors, DFF45 and poly (ADP-ribose) polymerase-1 (PARP-1), was also increased and followed a dose-dependent manner. All of these findings suggest that the apoptosis of PC-3 cells may proceed through the intrinsic mitochondria pathway.

KEYWORDS: *Gynostemma pentaphyllum*, prostate cancer cell, flavonoid, saponin, apoptosis, cell cycle

INTRODUCTION

Gynostemma pentaphyllum, a popular Chinese herb widely used in Taiwan and China, has been reported to be effective against chronic disease like liver cancer,[1,2] which may be attributed to the presence of several functional components such as flavonoids and saponins. *G. pentaphyllum* is also named as "cheap ginseng" and has received greater attention than ginseng, a valuable medicinal herb widely used throughout the world, in the past decade.[3] However, the effect of bioactive compounds in *G. pentaphyllum* on prevention or possible treatment of chronic disease still remains uncertain. In view of the impact of *G. pentaphyllum* on human health, the effects of flavonoids and saponins on inhibition of some other types of cancer cells need to be further explored.

Kim et al.[4] studied the effect of ginseng saponin RK_1 on inhibition of liver cancer cell HepG2 and found that with RK_1 dose at 100 μM, about 95% of HepG2 cell can undergo apoptosis through elevation of caspase-3 activity, an enzyme responsible for apoptosis execution. In another study, Wang et al.[5] isolated 25-hydroxyprotopanaxadiol (25-OH-PPD) and 25-hydroxyprotopanaxatriol (25-OH-PPT) from *Panax ginseng*, and a dose-dependent inhibition on prostate cancer cell lines PC-3 and LNCaP was observed for the former through enhanced expression of the pro-apoptotic proteins including P21, P27, and Bax as well as activation of caspase-3. Likewise, a dose- and time-dependent antiproliferation on tongue cancer cells SCC-4 by gypenoside, a component of *G. pentaphyllum*, through inhibition in expression of the antiapoptotic proteins Bcl-2 and Bcl-x1 and increase in expression of Bax was reported by Chen et al.[6] In addition, the administration of *G. pentaphyllum* extract into guinea pigs was protective against pitressin-induced coronary spasm, arrhythmias, and pressor response,[7] while a dose-dependent reduction in total cholesterol, triglyceride, and low-density lipoprotein in the Zucker fatty rat was reported by Megalli et al.[8] Also, the liver protective effect was shown after administration of compound K, produced from ginsenosides of *P. ginseng*, to mice.[9]

Similar to saponins, flavonoids represent a vital class of functional components in *G. pentaphyllum*. Approximately 6500 flavonoids have been characterized in nature, which are widely distributed in various fruits and vegetables.[10] Flavonoids are composed of two benzene rings and one pyrene ring with most being present in glycosidic form in plants and can be divided into six classes of flavone, flavonol, flavanol, flavanone, isoflavone, and anthocyanidin.[1] It has been well documented that flavonoids possess pivotal biological activities like anti-inflammation and anticancer. For instance, a citrus fruits flavonoid, hesperidin was effective in inhibiting expression of inducible nitric oxide synthase (iNOS) and production of nitrogen dioxide and prostaglandin E2 (PGE_2), which may lead to the anti-inflammatory and antitumorigenic function.[11] Likewise, the TPA-induced skin inflammation in mice was reduced after wogonin treatment through reduction of PGE_2 concentration and a decrease in mRNA levels of COX-2 and TNF-α expression.[12] Also, the citrus flavonoids have been demonstrated to be protective against atherosclerosis, mutagenicity, tumor cell proliferation, and tumor

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development.[13] In a previous study, we reported the antiproliferation of *G. pentaphyllum* flavonoids and saponins on hepatoma cell.[1] However, the antiproliferative activity and mechanism of prostate cancer cell as affected by flavonoids and saponins from *G. pentaphyllum*, a different variety grown in Taiwan, remains unknown. The objectives of this study were to isolate flavonoids and saponins from *G. pentaphyllum* by open-column chromatography, and the antiproliferation and apoptosis mechanism of prostate cancer cell PC-3 was elucidated.

MATERIALS AND METHODS

G. pentaphyllum, a specific variety grown in Taiwan, was procured from a local drug store in Taipei, Taiwan. The saponin standard, ginsenoside Rb_3, was from Extrasynthese (Genay, France), while ginsenoside Rd and protopanaxatriol were from LKT Laboratories Inc. (St. Paul, MN). The flavonoid standards, rutin and kaempferol, were from Sigma (St. Louis, MO), while kaempferol-3-*O*-rutinoside was from Chromadex (Santa Ana, CA). The adsorbent Cosmosil $75C_{18}$−OPN (particle size 75 μm) was from Nacalai Co. (Kyoto, Japan). The high-performance liquid chromatography (HPLC)-grade solvents including methanol, acetonitrile, and ethanol were from Lab-Scan Co. (Gliwice, Poland). Formic acid was from Riedel-de Häen Co. (Seelze, Germany). Deionized water was obtained using a Milli-Q water purification system (Millipore Co., Bedford, MA).

F-12K medium, 0.125% trypsin-EDTA, and penicillin-streptomycin were from Gibco Co. (CA). The trypan blue stain (0.4%) was from Invitrogen Co. (Carlsbad, CA). The fetal bovine serum (FBS) was from Hyclone Co. (Fremont, CA). The MTT reagent 3-(4,5-dimethylthiazol)-2, 5-diphenyltetrazolium bromide and ribonuclease (RNase A) were from Sigma. The Annexin-V, propidium iodide (PI), and caspase-3 assay kit were from BD Bioscience (San Diego, CA). SDS (sodium dodecyl sulfate), TEMED, Tween 20, 40% acrylamide/Bis, and DMSO were from J. T. Baker Co. (Phillipsburg, NJ). The primary antibody including anticyclin B, anti-DFF45, and anticytochrome *c* were from BD Bioscience. Antiactin was from Sigma, and anti-Bcl-2, anti-Bcl-xl, and anticaspase-3 were from Epitomics Co. (Burlingame, CA). The secondary antibody goat antimouse IgG-HRP and rabbit antimouse IgG-HRP were from Chemicon Co. (Temecula, CA). Human prostate cancer cell line PC-3 was from Taiwan Food Industry Development Institute/National Health Research Institute (Hsinchu, Taiwan).

A Gemini C18 column (250 mm × 4.6 mm i.d., particle size 5 μm) from Phenomenex Co. (Torrance, CA) was used to separate saponins and flavonoids.

Instrumentation. The Agilent 1100 series HPLC system contains a G1312A binary pump, G1311A quaternary pump, a G1316A column controller, a G1379A degasser, and a G1315B photodiode array detector. An Agilent 6130 quadrupole mass spectrometer with multimode ion source and a 1200 cap series 6510 Q-TOF LC/MS/MS was also used. The Sorvall RC5C high-speed centrifuge was from Du Pont (Wilmington, DE). The sonicator (2210R-DTH) was from Branson (Danbury, CT). The laminar flow (VCM-620) was from Jau-Hsing Co. (Taipei, Taiwan). The inverted microscope (TS-100) was from Nikon (Tokyo, Japan). The enzyme-linked immunosorbent assay (ELISA) reader (model Mulitiskan) was from Thermo Co. (Fremont, CA). The flow cytometer (CyFlow ML) was from Partec Co. (Munster, Germany). The spectrophotometer (DU 6408) was from Beckman Co. (Fullerton, CA).

Extraction of Flavonoids and Saponins. A method based on Kao et al.[14] was modified to extract flavonoids and saponins from *G. pentaphyllum*. Briefly, a 30 g powder sample of *G. pentaphyllum* leaves was mixed with 150 mL of ethanol, followed by shaking the mixture at 60 °C for 3 h and then centrifuging at 6500 rpm for 30 min at 25 °C. The supernatant was collected and filtered through a 0.22 μm membrane filter to obtain crude extract of flavonoids and saponins, after which 20 μL was injected for HPLC-MS analysis and 2 mL was collected for preparation of flavonoids and saponins by open-column chromatography. After HPLC-MS analysis, the total flavonoid and saponin contents in *G. pentaphyllum* were about 8.1 and 223 mg/g based on dry weight, respectively.

Preparation of Flavonoids and Saponins. A method as described by Tsai et al.[1] was modified to prepare flavonoids and saponins from *G. pentaphyllum* by open-column chromatography. Initially, 5 g of adsorbent Cosmosil $75C_{18}$−OPN was poured into a glass column (280 mm × 15 mm i.d.), which was previously activated with 100 mL of deionized water and 100 mL of ethanol. Prior to loading, 2 mL of crude extract was evaporated to dryness under nitrogen and dissolved in 1 mL of 0.1% formic acid solution. Then, the 1 mL solution was poured into the column, and 60 mL of deionized water was added to remove the impurities with flow rate at 5 mL/min, followed by eluting flavonoids with 60 mL of ethanol−water (30:70, v/v) and saponins with 20 mL of 100% ethanol. Both fractions were collected separately and evaporated to dryness under vacuum, followed by dissolving in 5 mL of ethanol, filtering through a 0.22 μm membrane filter, and injecting 20 μL into HPLC.

HPLC-MS Analysis of Flavonoids. An HPLC-MS method as described by Kao et al.[14] was employed to separate the various flavonoids in flavonoid fraction. A gradient mobile phase of 0.1% formic acid solution (A) and methanol (B) were used to separate eight flavonoids within 45 min with a Gemini C18 column (250 mm × 4.6 mm i.d., particle size 5 μm) and flow rate at 1 mL/min, column temperature at 35 °C, and detection wavelength at 280 nm, followed by mass spectra determination. The identification was based on comparison of retention times, UV spectra characteristics, mass spectra, and fragmentation patterns with reference standards and those in the literature.[14] For quantitation, five concentrations of caffeic acid, rutin, and kaempferol-3-*O*-rutinoside were prepared in methanol separately, followed by adding internal standard kaempferol to each solution. Then, the three standard curves were prepared by plotting the concentration ratio against its area ratio, with the regression equations being $y = 1.124x + 0.082$, $y = 0.7642x - 0.066$, and $y = 1.2485x - 0.2211$, respectively, and R^2 being all higher than 0.99. The various flavonoids were then quantified using a formula as described by Kao et al.[14]

HPLC Analysis of Saponins. Similar to flavonoids, the various saponins in saponin fraction prepared from *G. pentaphyllum* were separated with a Gemini C18 column (250 mm × 4.6 mm i.d., 5 μm particle size) and a gradient solvent system of 0.1% formic acid solution (A) and acetonitrile (B) with flow rate at 1 mL/min and detection by ELSD, followed by mass spectra determination and identification based on comparison of retention times and mass spectra, and fragmentation patterns of unknown peaks with authentic standards and those in the literature.[14] For quantitation, five concentrations of ginsenosides Rd and Rb_3 were prepared in methanol separately, followed by adding internal standard protopanaxatriol to each solution. Then, both standard curves of ginsenosides Rd and Rb_3 were obtained with the regression equations being $y = 1.2564x - 0.4044$ and $y = 0.5411x - 0.3098$, respectively, and R^2 being higher than 0.99. The various saponins in saponin fraction were then quantified using a formula as described by Kao et al.[14]

MTT Assay. Human prostate cancer cell line PC-3 was cultured in a F12-K medium containing 7% FBS and 100 U/mL of penicillin-streptomycin. Cells were incubated under 5% CO_2 at 37 °C, and the medium was replaced every 2 days to maintain normal cell growth. After growth to a high density at about 90% confluence, cells were washed with PBS solution twice, and 0.25% trypsin-EDTA solution was added for reaction at 37 °C for 2 min, after which the medium was added for neutralization. Then, cells were collected with a pipet, and fresh medium was added. Next, cells were seeded in a 24-well plate with each well

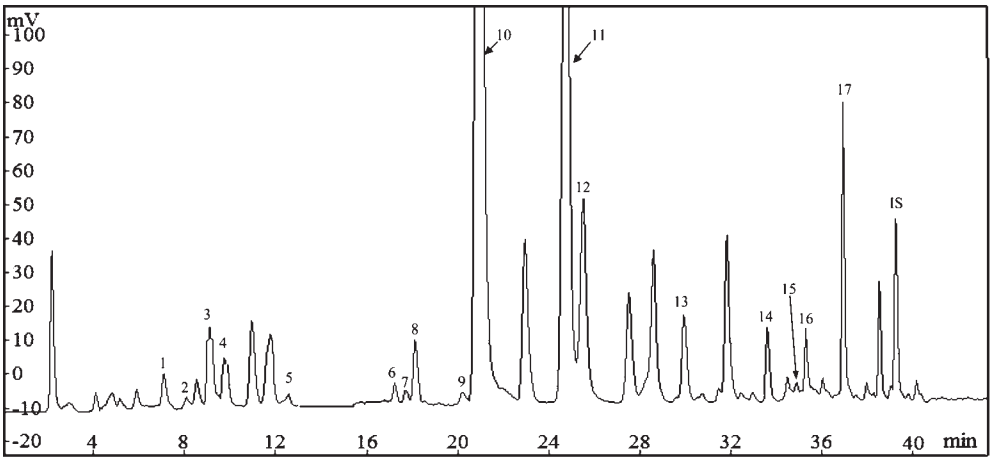

Figure 1. HPLC chromatogram of various saponins in saponin fraction from *G. pentaphyllum*. Chromatographic conditions are described in the text.

containing 2.5×10^5 cells and incubated for 24 h for cell attachment, after which the medium was removed and replaced with fresh medium containing five doses of 30, 50, 80, 100, and 150 μg/mL of flavonoid fraction or five doses of 10, 30, 50, 80, and 100 μg/mL of saponin fraction and incubated for another 72 h. Then, 50 μL of MTT solution (0.5 mg/mL) was added to each well and incubated at 37 °C for 3 h, followed by adding 200 μL of DMSO for reaction for 15−30 min to dissolve crystal, collecting 150 μL and transferring to a 96-well plate, and measuring the absorbance at 570 nm with an ELISA reader. Duplicate analyses were carried out for each dose, with both relative cell survival rate and IC_{50} being determined.

Cell Cycle Analysis. Human prostate cancer cell line PC-3 (1×10^6) was seeded in a 10 cm culture plate and incubated for 24 h, after which the medium was removed and replaced with fresh medium containing 30, 50, 80, 100, and 150 μg/mL of flavonoid fraction or 10, 30, 50, 80, and 100 μg/mL of saponin fraction and incubated for another 48 h. Then, cells were washed with PBS, and 0.25% trypsin-EDTA was added to detach cells for collection with PBS. Next, cells were centrifuged at 1500 rpm (4 °C) for 5 min, and the supernatant was collected, and PBS containing precooled 70% ethanol was added to fix cells. Again, the cell solution was centrifuged at 1500 rpm (4 °C) for 5 min, followed by removing supernatant, washing with PBS three times, adding PI buffer (0.1% Triton X-100, 0.2 μg/mL RNase A, and 20 μg/mL PI) for staining, reacting at 25 °C for 30 min, and analyzing cell cycle with a flow cytometer.

Cell Apoptosis Analysis (Annexin V and PI Staining). PC-3 cells (1×10^6) were incubated in a 10 cm culture plate for 24 h, after which 30, 50, 80, 100, and 150 μg/mL of flavonoid fraction or 10, 30, 50, 80, and 100 μg/mL of saponin fraction was added and incubated for 48 h, followed by washing with PBS, and cells were detached as described above. Then, the cell solution was collected in a 15 mL centrifuged tube, washed with cold PBS twice, and centrifuged at 1500 rpm (4 °C) for 5 min. The supernatant was removed, and 0.5 mL of PBS was added to disperse cells in a microcentrifuged tube, followed by centrifugation at 200*g* (4 °C) for 5 min, removal of the supernatant, addition of 0.1 mL of 1× binding buffer (0.01 M Hepes/NaOH (pH 7.4), 0.14 M NaCl, and 25 mM $CaCl_2$), addition of 5 μL of Annexin V-FITC and 5 μL of PI staining agent, mixing thoroughly, reaction at 25 °C in the dark for 15 min, and analysis of cell apoptosis with a flow cytometer.

Western Blot Analysis. PC-3 cells (1×10^6) were cultured in a 10 cm plate and incubated for 24 h, after which the medium was removed and replaced with fresh medium or medium containing 30, 50, 80, 100, and 150 μg/mL of flavonoid fraction or 10, 30, 50, 80, and 100 μg/mL of saponin fraction. After 48 h of incubation, cells were detached by scratching with a scraper, followed by washing of cells with PBS three times, centrifugation, addition of lysis buffer, and sonication to disrupt cells for protein release. Then, the cell solution was centrifuged at 14000 rpm (4 °C) for 10 min, and the supernatant was collected in a 1.5 mL centrifuge tube for protein quantitation. Seven concentrations of 0, 25, 50, 125, 250, 500, and 1000 μg/mL of protein standard (bovine serum albumin) were prepared, and a portion (25 μL) was collected and mixed with 500 μL of dye reagent (Bio-Rad protein assay), after which the mixture was reacted at 37 °C for 30 min, and the absorbance was measured at 562 nm. The protein standard curve was thus obtained by plotting concentration against area, and the protein sample concentration was calculated from regression equation of the standard curve.

For electrophoresis, protein sample was prepared in 1× SDS gel-loading buffer containing 50 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. The premade gel containing 10% SDS-polyacrylamide was placed in a tank, and cell protein was separated under 200 V for 30 min, after which cell protein in transfer buffer (40 mM glycine, 50 mM Tris base, 0.04% SDS, and 20% methanol, pH 8.3) was transferred to nitrocellulose membrane under 100 V for 1 h at 4 °C. Then, the membrane was stained with Ponceau S and washed with deionized water, followed by soaking in a buffer containing 5% skim milk for 20 min to remove background noise. Next, the membrane was washed with TBS-T solution containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 to remove unattached protein, and primary antibody was added and

Table 1. Contents (μg/mL) of Various Flavonoids in Flavonoid Fraction from *G. pentaphyllum*

peak no.	flavonoids[a]	content (μg/mL)[b]
1	caffeic acid	2.5 ± 0.1
2	quercetin-di(rhamno)-hexoside	15.3 ± 0.7
3	quercetin-rhamno-hexoside	51.2 ± 5.3
4	kaempferol-rhamno-hexoside	55.2 ± 1.3
5	kaempferol-rhamno-hexoside	101.8 ± 0.2
6	rutin	16.6 ± 0.7
7	kaempferol-rhamno-hexoside	3.8 ± 0.1
8	kaempferol-3-*O*-rutinoside	4.2 ± 0.2
IS	kaempferol	
	total	250.6

[a] Identity based on LC-MS-MS data.[1] [b] Average of duplicate analyses ± standard deviations.

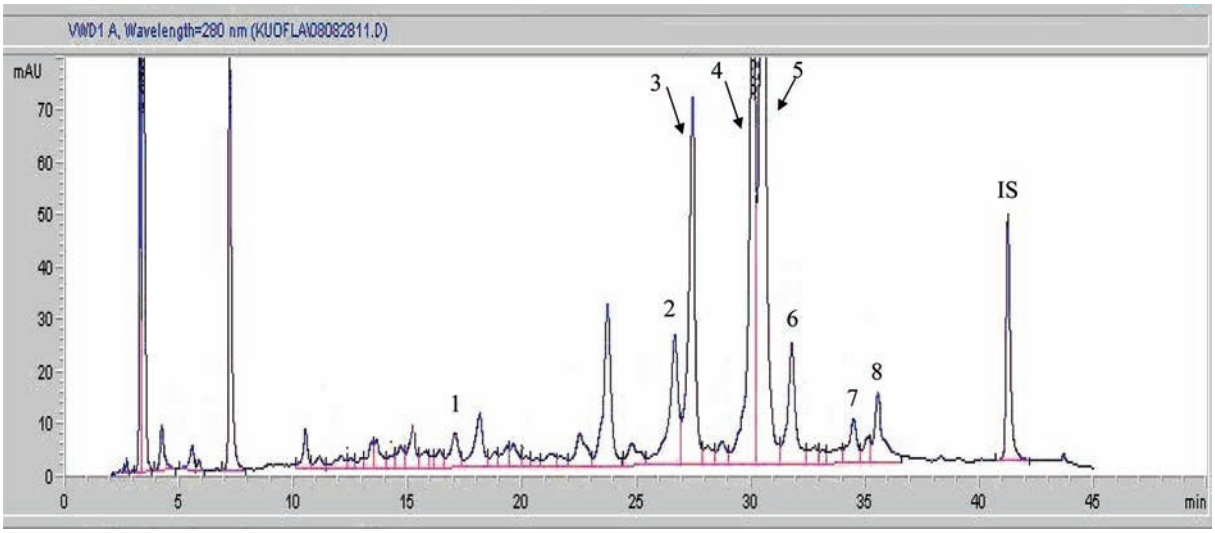

Figure 2. HPLC chromatogram of various flavonoids in flavonoid fraction from *G. pentaphyllum*. Chromatographic conditions are described in the text.

reacted for 1 h at room temperature for protein conjugation. Again, the TBS-T solution was added to wash for 5 min for five times, and secondary antibody containing horseradish peroxidase was added and reacted at room temperature for 1 h for protein conjugation. After it was washed with TBS-T solution for 5 min for five times, the supersignal west dura luminol/enhancer was added for chemiluminescence emission. Finally, the classic autoradiograph film was used to press for image expression by film developer and for detection of protein signal and intensity.

Statistical Analysis. Duplicate analyses were performed, and all of the data were subjected to analysis of variance (ANOVA) and Scheffe's multiple-range test for mean comparison ($p < 0.05$) based on SPSS software system.[15]

RESULTS

HPLC-MS Analysis of Flavonoid and Saponin Fractions. Following the same approach as described in a previous study,[1] a total of nine flavonoids were separated, and eight were identified (Figure 1 and Table 1), including caffeic acid (2.5 μg/mL), quercetin-di(rhamno)-hexoside (15.3 μg/mL), quercetin-rhamno-hexoside (51.2 μg/mL), kaempferol-rhamno-hexoside (55.2 μg/mL), kaempferol-rhamno-hexoside (101.8 μg/mL), rutin (16.6 μg/mL), kaempferol-rhamno-hexoside (3.8 μg/mL), and kaempferol-3-O-rutinoside (4.2 μg/mL), with the total amount being 250.6 μg/mL. Likewise, a total of 17 saponins were separated and identified (Figure 2 and Table 2), including Gyp LXI (7.0 μg/mL), Gyp-2 (16.0 μg/mL), Gyp LXI (0.2 μg/mL), Gyp XLII (0.6 μg/mL), GypLXIII (7.0 μg/mL), Gyp XXII (553.0 μg/mL), Gyp XXIII (348.8 μg/mL), Gyp IV (Ginsenoside Rb_3) (77.2 μg/mL), Gyp VIII (Ginsenoside Rd) (56.2 μg/mL), Gyp LVII (49.2 μg/mL), and Ginsenoside R_f or Gynoside B (49.0 μg/mL) and Gynoside A or C or E (113.8 μg/mL), with the total content being 1278.0 μg/mL.

MTT Assay. The inhibition effect of saponin and flavonoid fractions from *G. pentaphyllum* on PC-3 cell growth as determined by MTT assay is shown in Figure 3. A dose-dependent inhibition on PC-3 cells was found for both flavonoid and saponin fractions. With saponin fraction dose at 10 μg/mL, the relative cell survival rate of PC-3 was 87.9%, implying that a poor inhibition occurred at a low dose. However, following a rise in saponin fraction dosage, the relative cell survival rate declined significantly, and an 80% inhibition of PC-3 cells was attained at 100 μg/mL. Similarly, with flavonoid fraction treatment, a comparable inhibitory effect on PC-3 growth was observed, a slightly stronger inhibition of 47.7% (39.9% for saponin) at 30 μg/mL, while a comparable 58.5% (58.9% for saponin) at 50 μg/mL, and an 80% retardation was achieved at a high dose of 150 μg/mL. Comparatively, the flavonoid fraction was slightly more effective in antiproliferation of PC-3 cells at lower concentration (<50 μg/mL), whereas saponin fraction exhibited stronger effect at higher concentration (>50 μg/mL), as evidenced by the IC_{50} of 33.3 μg/mL for the former and 39.3 μg/mL for the latter, also by the maximal inhibition of 80% achieved at 100 μg/mL for the former and 150 μg/mL for the latter. This is why the doses of

Table 2. Contents (μg/mL) of Various Saponins in the Saponin Fraction from *G. pentaphyllum*

peak no.	gypenoside	content (μg/mL)[a]
1	Gyp LXI[b]	5.2 ± 1.3
2	Gyp LXI[b]	1.8 ± 0.2
3	Gyp-2	8.8 ± 0.9
4	Gyp-2	7.2 ± 0.2
5	Gyp LXI[b]	0.2 ± 0.1
6	Gyp XLII[b]	0.6 ± 0.2
7	Gyp LXIII	0.2 ± 0.1
8	Gyp LXIII	6.8 ± 0.3
9	Gyp XXII[b]	0.2 ± 0.1
10	GypXXII[b]	552.8 ± 6.2
11	Gyp XXIII[b]	348.8 ± 10.9
12	Gyp IV (ginsenoside Rb_3)[a]	77.2 ± 20.2
13	Gyp VIII (ginsenoside Rd)[a]	56.2 ± 19.1
14	Gyp LVII	49.2 ± 13.3
15	ginsenoside Rf or gynoside B[b]	0.2 ± 0.6
16	ginsenoside Rf or gynoside B[b]	48.8 ± 0.9
17	gynoside A or gynoside C or gynoside E[b]	113.8 ± 30.9
	total	1278.0

[a] Average of duplicate analyses ± standard deviations. [b] Probable identity based on LC-MS-MS data.[1]

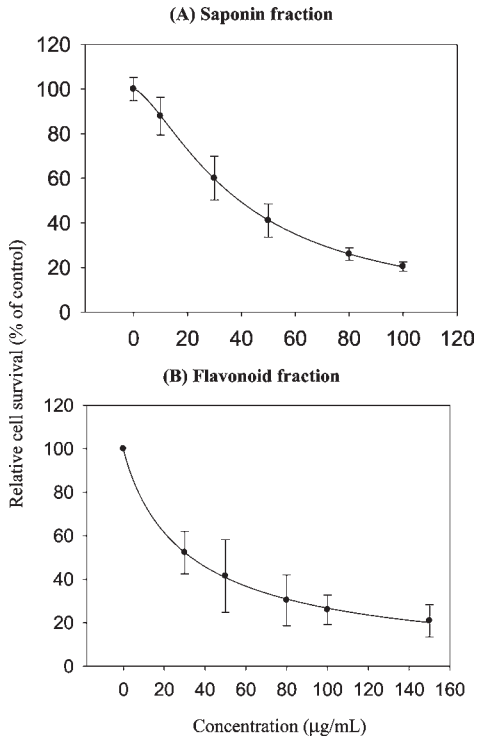

Figure 3. Inhibition effect of saponin and flavonoid fractions from *G. pentaphyllum* on PC-3 cell growth as determined by MTT assay. Data are shown as averages of duplicated analyses with indicated standard deviations.

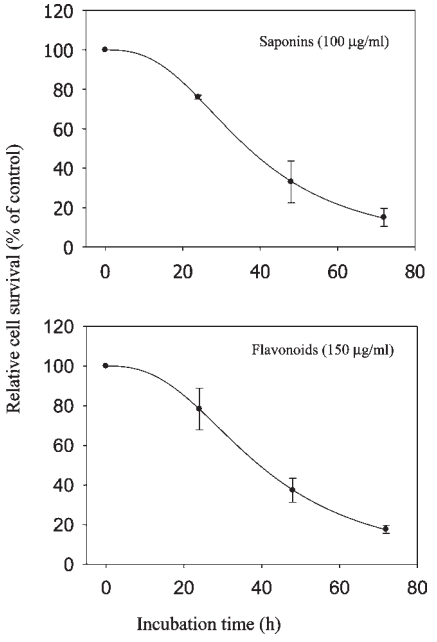

Figure 4. Inhibition effect of saponin and flavonoid fractions from *G. pentaphyllum* on PC-3 cell as affected by incubation time. Data are shown as averages of duplicated analyses with indicated standard deviations.

30 and 100 μg/mL for saponin fraction as well as 30 and 150 μg/mL for flavonoid fraction were selected for subsequent experiments.

Figure 4 shows the inhibition effect of saponin fraction (100 μg/mL) and flavonoid fraction (150 μg/mL) from *G. pentaphyllum* on PC-3 cells as affected by incubation time. Apparently, a time-dependent inhibition was observed for both saponin and flavonoid fractions. Under the same incubation time at 24 h, the relative cell survival rate was 75.8 and 78.3% for saponin and flavonoid fractions, respectively, indicating a comparable effective inhibition. The same trend followed over an incubation period from 24 to 72 h, with a maximum inhibition of 83.3% being reached for saponin fraction at 100 μg/mL and 82.3% for flavonoid fraction at 150 μg/mL in 72 h.

Cell Cycle Analysis. Figure 5 shows the effect of saponin and flavonoid fractions from *G. pentaphyllum* on cell cycle distribution of PC-3 cells. For all of the treatments, a dramatic dose-dependent modulation was observed as shown in the cell population ratios of sub-G0/G1 (RN1%), G0/G1 (RN2%), S (RN3%), and G2/M (RN4%) phases. As shown in Figure 5, a dose-dependent increase for the sub-G0/G1 accompanied by a decrease for G0/G1 phase, revealing cell apoptosis or necrosis may occur after flavonoid or saponin treatment. More specifically, with flavonoid dose at 30 μg/mL, the sub-G0/G1 ratio (5.62%) was 4.8-fold to that of the control (1.16%). On the contrary, the sub-G0/G1 ratio (4.36%) was only 1.8-fold to that of the control (2.49%) after saponin treatment at 30 μg/mL. Following a rise in dose to 100 μg/mL, the sub-G0/G1 ratio was raised to a plateau (7.16%) for saponin fraction but to a peak (26.68%) for flavonoid fraction, indicating a greater cell apoptosis or necrosis effect after flavonoid treatment when compared with saponin treatment. In contrast to sub-G0/G1, the G0/G1 ratio showed a sharp decline at low dose, followed by an increased trend at high doses of both saponin and flavonoid fractions. With saponin fraction at 10 μg/mL and flavonoid fraction at 30 μg/mL, the G0/G1 ratio was diminished to a larger extent than the control treatment by 58.72 and 27.42%, respectively. With the increment in both doses of saponin (from 10 to 100 μg/mL) and flavonoid (from 30 to 150 μg/mL) fractions, the G0/G1 ratio gradually stabilized to ~39 and ~48% (control ~67−71%), respectively. The reduction in G0/G1 ratio was coincided with variable ratio changes of S and G2/M phases after treatment with both saponin and flavonoid fractions. For the S phase, the ratio was raised to a peak of ~29 and ~24% at 30 μg/mL for both saponin and flavonoid fractions, respectively. However, with a raise in both doses of saponin (from 50 to 100 μg/mL) and flavonoid (from 50 to 150 μg/mL) fractions, the S ratios were alleviated to ~22 and ~10% (control ~7%), respectively. Like the S phase, the G2/M ratio displayed a more prominent increase following treatment with saponin as compared with flavonoid fractions. More elaborately, a peak of G2/M ratio was attained for saponin fraction at 10 μg/mL and flavonoid fraction at 30 μg/mL, which equaled 55.88 and 27.99%, respectively. This outcome revealed that saponin or flavonoid treatment could induce an arrest of PC-3 cell cycle at both S and G2/M phases. By comparison, the saponin treatment could induce a higher G2/M ratio than the flavonoid treatment under the same dosage. Alleviation of G2/M arrest was observed over the dose range from 30 to 100 μg/mL for saponin fraction and from 50 to 150 μg/mL for flavonoid fraction. This alleviation of cell cycle arrest also coincided with the recovery of the G0/G1 ratio following a high dose of saponin or flavonoid treatment, as well as the increment in sub-G0/G1 ratio after high dose, especially 150 μg/mL of flavonoid treatment.

Annexin-V Analysis. The Annexin-V/PI staining method can be used to distinguish between cell apoptosis and necrosis.

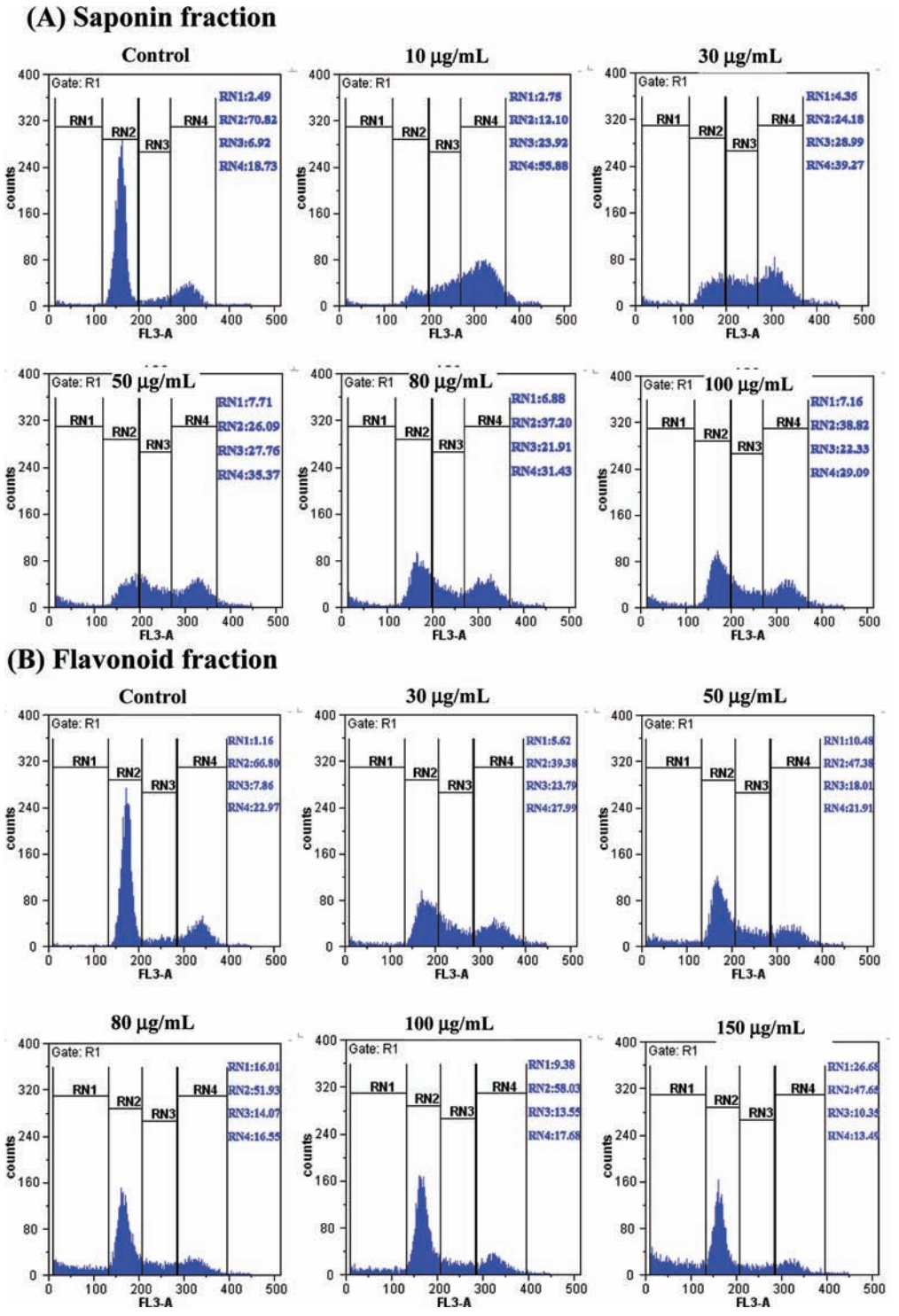

Figure 5. Effects of saponin and flavonoid fractions from *G. pentaphyllum* on cell cycle distribution of PC-3 cells. Cell population percentages of sub-G0/G1, G0/G1, S, and G2/M phases are indicated as RN1, RN2, RN3, and RN4%, respectively.

During apoptosis, cell membrane can be overturned to translocate phosphatidylserine (PS) for conjugation with Annexin-V to show the Annexin-V positive phenomenon, which can be detected with a flow cytometer based on fluorescence signal produced in FL1 channel by FITC-labeled Annexin-V. In addition, the loss of membrane integrity accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Disruption of cell membrane causes conjugation of PI with DNA to show PI positive phenomenon, which can also be detected with a flow cytometer based on fluorescence produced in the FL3 channel. As a result, cells exhibited the Annexin-V/PI positive phenomenon after incorporation of both Annexin-V and PI at the same time during the late period of apoptosis and necrosis.

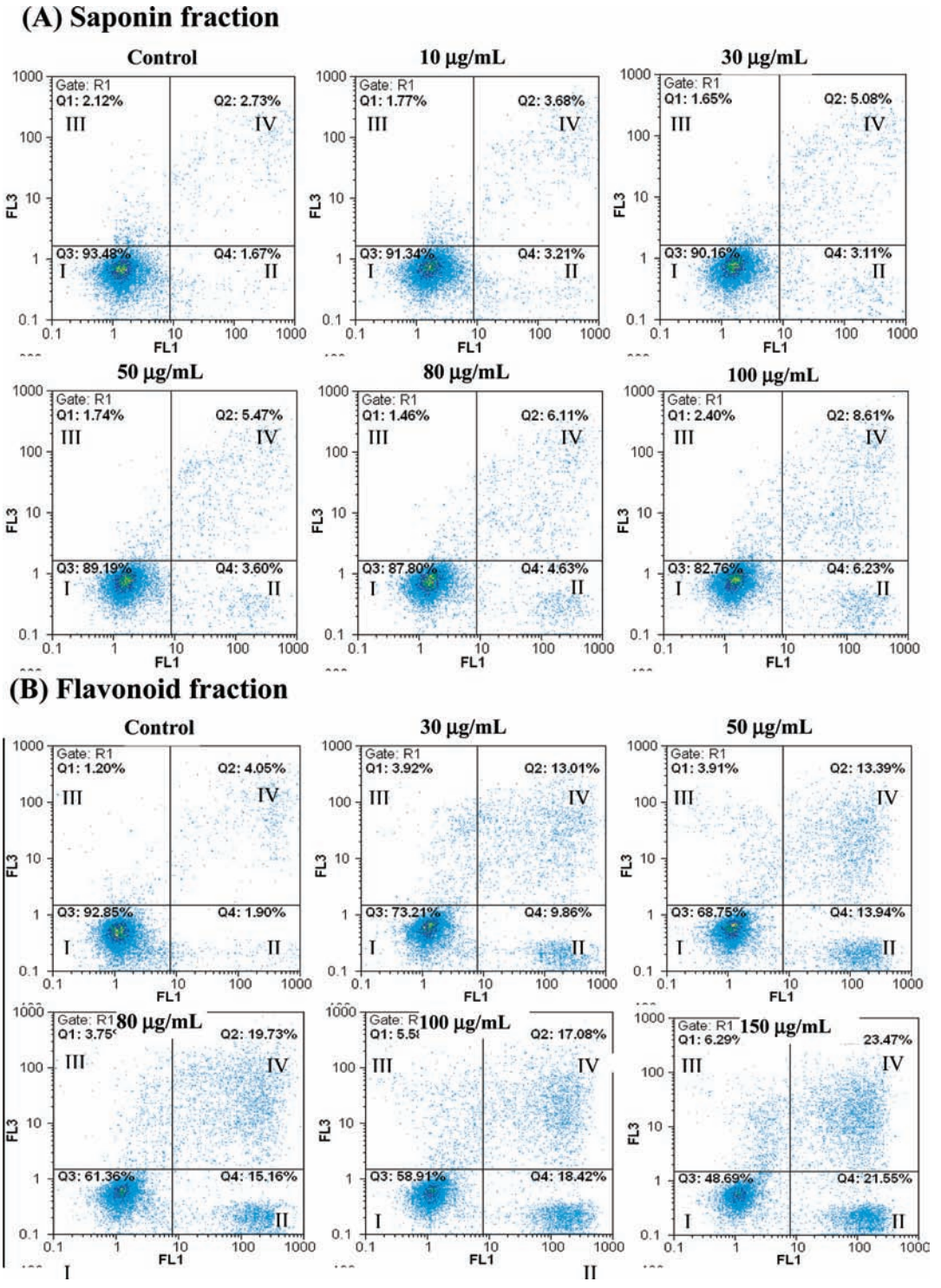

Figure 6. Annexin-V analysis result of PC-3 cells by a flow cytometer as affected by saponin (A) and flavonoid (B) fractions. I, normal; II, annexin V positive; III, PI positive; and IV, Annexin/PI positive.

The Annexin-V analysis results of PC-3 cells by a flow cytometer as affected by flavonoid and saponin fractions are shown in Figure 6, in which area I denotes normal cell distribution, area II early apoptotic cell distribution (Annexin V positive), and area IV late apoptotic and necrotic cell distribution (Annexin/PI positive). After flavonoid treatment for 48 h, PC-3 cells were migrated to the Annexin V positive area II by following a dose-dependent response, ~10% at 30 μg/mL to ~22% at 150 μg/mL, implying that an early apoptosis did occur. This early apoptotic event also resulted in increasing ratio in the Annexin/PI positive area IV, ~13% at 30 μg/mL to ~23% at 150 μg/mL, indicating that PC3 cells may undergo transition from early to late apoptotic stage following flavonoid treatment. Only minimal (~6%) PC-3 cells were migrated to the Annexin negative/PI positive area III even if

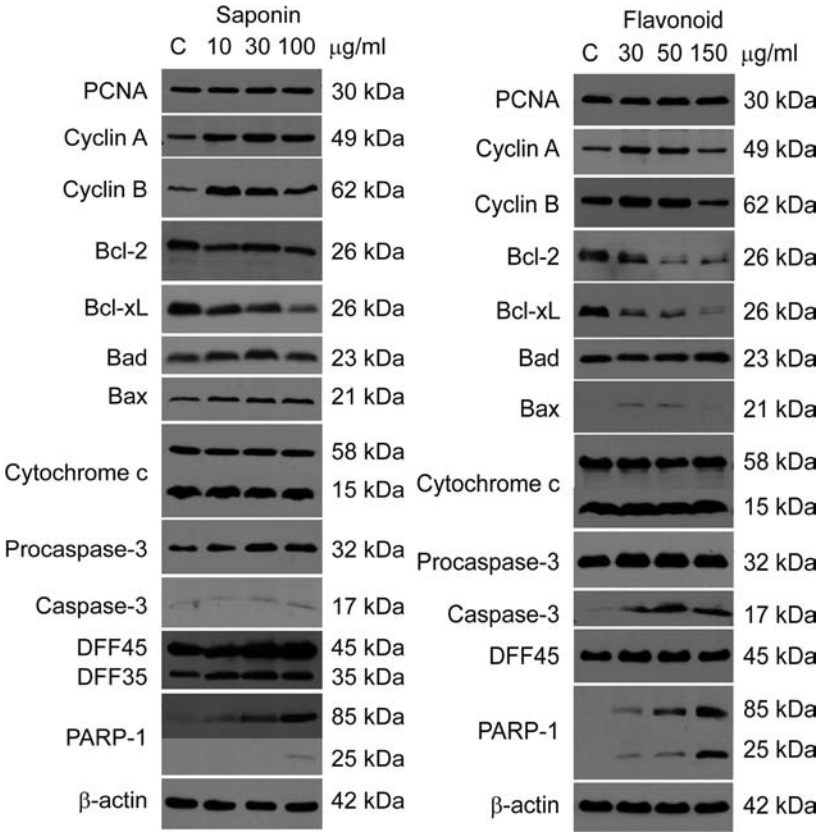

Figure 7. Expression of apoptotic proteins in PC-3 cells as affected by saponin and flavonoid fractions from *G. pentaphyllum*. Data were analyzed with gene tools, and β-actin was used as an internal control.

the flavonoid fraction dose was raised to 150 μg/mL. On the contrary, after saponin treatment for 48 h, PC-3 cells exhibited only minimal migration into the Annexin V positive area II (~3% at 10 μg/mL to ~6% at 100 μg/mL) and the Annexin/PI positive area IV (3.68% at 10 μg/mL to 8.61% at 100 μg/mL) following a dose rise from 10 to 100 μg/mL. As compared to flavonoid treatment, only a relative less portion of PC-3 cells underwent both early and late apoptosis after saponin treatment, implying that saponin was less effective to induce apoptosis in PC-3 cells. Nevertheless, a significant portion of PC-3 cells was observed to undergo apoptosis after saponin treatment at a dose of 100 μg/mL for more than 48 h, 16.22 and 19.18% of PC-3 cells migrated to the Annexin V positive area II, as well as 20.69 and 30.79% migrated to the Annexin/PI positive area IV after 60 and 72 h, respectively (data not shown).

Expression of Proteins Associated with Cell Cycle Regulation and Apoptosis. It has been well established that cyclins control the progression of the cell cycle by activating cyclin-dependent kinase (CDK) enzymes.[29] For example, cyclin A/CDK2 and cyclin B/CDK1 complexes are active and are required to progress through S to G2 phase and from G2 to M transition during cell cycle, respectively. In addition, proliferating cell nuclear antigen (PCNA) is a processivity factor for DNA polymerase δ in eukaryotic cells and is originally identified as an antigen expressed during the S phase of the cell cycle.[30] As low concentrations of both saponin and flavonoid fractions could induce cell cycle arrest at both S and G2/M phases, the expression of PCNA and cyclins A and B need to be investigated. Furthermore, the expression of apoptotic proteins, such as Bcl-2 family of apoptosis regulator proteins, cytochrome *c*, caspase-3, and other downstream apoptotic effectors, in PC-3 cells as affected by saponin and flavonoid fractions from *G. pentaphyllum*, were also analyzed and compared against β-actin as an internal control. As shown in Figure 7, after saponin fraction treatment (10−100 μg/mL), increments in both cyclins A (49 kDa) and B (62 kDa) but not PCNA (30 kDa) expression in PC-3 cells were observed. The expression of cyclins A and B reached the maximal level at 30 and 10 μg/mL, respectively, which is consistent with our cell cycle distribution results that the peak S and G2/M arrest occurred after treatment with corresponding concentrations of saponin fractions (Figure 5A). A dose-dependent expression decline of antiapoptoic proteins like Bcl-2 (26 kDa) and Bcl-xL (26 kDa) was shown with a maximal reduction at a saponin concentration of 100 μg/mL. On the contrary, a slightly increased expression occurred for the expression of pro-apoptoic proteins like Bad (23 kDa) and Bax (21 kDa). Accordingly, both pro-apoptotic and antiapoptotic proteins (Bcl-2 family) should be balanced under normal physiological conditions; however, the balance can be broken down to induce cell death by some other factors such as the presence of toxic compounds. Moreover, the inhibition of expression of antiapoptotic proteins could lead to cytochrome *c* release from mitochondria into cytoplasm for activation of caspase-9 and caspase-3 to induce apoptosis.[16] From Figure 7, although no obvious changes in the amounts of both mitochondrial cytochrome *c* monomer (15 kDa) or cytoplasmic polymeric form (58−60 kDa) were detected, it can be clearly shown that the expression of both procaspase-3 and caspase-3 was elevated in a dose-dependent manner, demonstrating

that the PC-3 cell apoptosis may proceed through the intrinsic mitochondrial pathway. Additionally, the expression of the caspase-3 substrate and downstream apoptotic effectors like DNA fragmentation factor (DFF 45; 45 kDa) and its degradation product DFF35 (35 kDa) as well as cleaved 85 and 25 kDa poly (ADP-ribose) polymerase-1 (PARP-1; 116 kDa) followed a dose-dependent increase, illustrating again that the caspase-3 activation did occur during apoptosis. Similar protein expression results were also observed on PC-3 cells after flavonoid fraction treatment (Figure 7B). However, it is noteworthy that the dose-dependent alterations among the cell cycle protein expression were more pronounced following saponin fraction treatment, whereas the expression of apoptotic protein exhibited more prominent dose-dependent changes among PC-3 cells treated with flavonoid fraction, in general.

DISCUSSION

As mentioned in the preceding section, both saponin and flavonoid fractions extracted from *G. pentaphyllum* are effective in antiproliferation of prostate cancer cell PC-3. However, even though the IC_{50} was comparable for saponin and flavonoid fractions, the former exhibited maximal antiproliferation of prostate cancer cell PC-3 at 100 μg/mL, which was lower than the latter (150 μg/mL). Most of previous studies regarding *G. pentaphyllum* have focused on the effects of saponin standards or extracts. In addition to prostate cancer cell, the growth of hepatoma cell lines including Hep3B, Huh 7, and HA22T/VGH was completely retarded after treatment of gypenoside at 600 μg/mL, a kind of saponin from *G. pentaphyllum*.[17] Likewise, a complete inhibition of lung cancer cell A549 when treated with *G. pentaphyllum* gypenoside at 150 μg/mL was observed by Lu et al.[18] Comparatively, in our study, the saponin dose required to inhibit PC-3 cell growth was at least comparable or better than that reported above,[17,18] probably caused by the degree of saponin purification. In our experiment, the saponin fraction was purified through open-column chromatography and, thereby, may possess a better antiproliferation effect. Nonetheless, the difference in cell line may also account for this effect; that is, the prostate cancer cell PC-3 may be more liable to cell cycle arrest and/or apoptosis after saponin treatment than hepatoma cell and lung cancer cell. Of the various saponins in saponin fraction, gypenoside was present in the largest amount (Table 2), which should contribute greatly to the antiproliferative activity of PC-3 cells.

For cell cycle analysis, PC-3 cells were arrested at both S and G2/M phases after saponin treatment. On the contrary, the cell cycle of both prostate cancer cell lines PC-3 and LNCaP was shown to arrest at G1 phase when treated with ginsenoside, a kind of saponin isolated from *P. ginseng*.[5] Similarly, the cell cycle of hepatoma cell lines HepG2 and HA22T/VGH was arrested at the G0/G1 phase when subjected to treatment of saponin extract (200−500 μg/mL) from *G. pentaphyllum*.[16] However, for the other cancer cell lines, after treatment of *Panax notoginseng* saponin extract at 500 μg/mL, the human colorectal cancer cells SW480 were arrested at S and G2/M phases, which was postulated to be due to the major saponins in notoginseng, including notoginsenoside R_1 and ginsenosides Rb_1, Rb_3, and Rg_1.[19] All of these outcomes suggested that the arrest of cell cycle at S and G2/M phases may be varied depending on difference in cell line as well as saponin variety and purity. As indicated before, the checkpoints of S and G2/M phase are mainly regulated by expression of cyclin A/CDK2 and cyclin B/CBK1 complexes, respectively. It is quite possible that the arrest of PC-3 cell cycle at S and G2/M phases can be affected by cyclins A and B expression and/or modification. In a study dealing with experimental therapy of prostate cancer *in vitro* with 25-hydroxyprotopanaxadiol (25-OH-PPD), a kind of saponin in *P. ginseng*, Wang et al.[5] reported that a portion of PC-3 cells (8.24%) underwent early apoptosis after 4 h of treatment at 100 μg/mL. However, following a rise in dose to 200 μg/mL, the population of early apoptotic PC-3 cells was raised to 42.6%. This result is different from our finding, as shown by a much less population (~6%) of early apoptotic PC-3 cells when treated with saponin fraction at 100 μg/mL for up to 48 h. Nonetheless, we did observe significant early and late apoptotic cell population after treatment for 60 (16.22%) and 72 h (19.18%). Apparently, the saponin variety may be more effective on cell cycle arrest instead of apoptosis, and the difference in incubation time may also account for this effect.

More importantly, in our experiment, the amounts of both procaspase-3 and activated caspase-3 were increased after treatment with the saponin fraction from *G. pentaphyllum* through a decrease in expression of the antiapoptotic proteins Bcl-2 and Bcl-xl and increase in expression of pro-apoptotic proteins Bad and Bax, accompanied by a rise in the levels of caspase-3 cleaved DFF-45 fragment (DFF-35), as well as cleaved 85 and 25 kDa PARP-1 fragments and finally to apoptosis. A similar phenomenon was also observed by Wang et al.,[5] reporting that the incorporation of 25-OH-PPD could induce a decline in Bcl-2 expression for release of cytochrome *c* from mitochondria to activate caspase-3, resulting in PARP degradation and apoptosis of both PC-3 and LNCaP cells. Similar outcomes were found for the antiproliferation effect of gypenoside on tongue cancer cell SCC-4[6] and lung cancer cell A-549.[18] The lack of apparent cytochrome *c* translocation in our experiment may require more sophisticated subcellular fractionation technique for further in-depth investigation. Nevertheless, the extrinsic pathway (the death receptor pathway) leading to apoptosis as affected by saponins from *G. pentaphyllum* needs to be further investigated.

Like saponins, the efficiency of flavonoids in inhibiting growth of cancer cells through cell cycle arrest at G2/M phase has been extensively studied. Six flavonoids, including quercetin, luteolin, chrysin, kaempferol, apigenin, and myricetin, were effective in inhibiting human esophageal cancer cell OE33 and KYSE-510 through induction of G2/M phase arrest during cell cycle, down-regulation of cyclin B1, and up-regulation of caspase-9 and caspase-3, leading to mitochondria-mediated apoptosis.[20,21] Likewise, after treatment of kaempferol-7-*O*-D-glucoside isolated from *Smilax china* L. rhizome at 40 μg/mL, the arrest of HeLa cells (human cervix carcinoma cell) at G2/M phase as well as up-regulation of Bax and down-regulation of Bcl-2 were observed, indicating that the apoptosis may proceed through the mitochondria pathway.[22] In another study dealing with the effect of soybean isoflavone on prostate cancer cells PC-3 and LNCaP, both doses of 30 and 50 μg/mL of genistein were effective in antiproliferation through arrest of cell cycle at the G2/M phase, as well as a decline in cyclin B1 expression and a rise in P53 expression.[23] Similarly, our data revealed both S and G2/M arrest of PC-3 cells accompanied by a dose-dependent transient elevation and followed by a reduction in cyclins A and B expression after flavonoid treatment. As kaempferol-rhamnohexoside was present in abundant quantity (157 μg/mL) in flavonoid fraction (Table 1), this flavonoid should play a key role in inhibiting growth of PC-3 cells. However, we have to point out that most reports are focused on the effect of flavonoids in

aglycone form on antiproliferation of cancer cells, which should be inadequate as most flavonoids are present in glycosidic form in plants. Moreover, several studies have demonstrated that flavonoid aglycones were superior to their corresponding flavonoid glucosides in antiproliferation of cancer cells,[24,25] which may explain why a lower antiproliferative activity of flavonoid fraction from *G. pentaphyllum* was observed in our study.

More specifically, when treated with flavonoid fraction from *G. pentaphyllum* at 150 μg/mL for 48 h, about 21.55 and 23.47% of PC-3 cells underwent early and late apoptosis, respectively, implying a dose-dependent antiproliferation by flavonoids. Similar to saponins, the Western blotting analysis also revealed that the flavonoid treatment could lead to a reduction in expression of Bcl-2 and Bcl-x1, as well as an increment in expression of procaspase-3 and activated caspase-3 as well as caspase-3 activated downstream effectors for apoptosis of prostate cancer cells PC-3. This phenomenon was also reported for some other types of cancer cells in several previous studies.[26] For instance, in a study dealing with the effect of quercetin on growth of lung cancer cell A549, a dose-dependent decrease in expression of Bcl-2 and rise in expression of caspase-3, caspase-7, and PARP was shown.[27] This report also suggested that in addition to inactivation of Akt-1 and alteration in expression of Bcl-2, the activation of MEK-ERK is essential for quercetin-induced apoptosis of lung cancer cell A549 to occur. In addition, with quercetin treatment, the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cytotoxicity and annexin V staining as well as PARP cleavage in human prostate cancer cell lines DU-145 and PC-3 were enhanced, as a result of activation of caspase-8, -9, and -3.[28] Further investigations are required to unveil cellular events that might initiate apoptotic signals after treatment.

In conclusion, the saponin and flavonoid fraction from *G. pentaphyllum* are both effective in antiproliferation of prostate cancer cell PC-3 by inducing an arrest of cell cycle at S and G2/M phases via modulating the expression of cyclins, as well as by inducing cellular apoptosis, resulting in a decline in expression of the antiapoptotic proteins and an increase of pro-apoptotic proteins, the caspase-3 and activated downstream substrate effectors by following a dose-dependent manner. Comparatively, the saponin fraction exhibited stronger effects on cell cycle modulation, whereas the flavonoid fraction induced mainly apoptosis of PC-3 cells, possibly through the mitochondria pathway.

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REFERENCES

(1) Tsai, Y. C.; Lin, C. L.; Chen, B. H. Preparative chromatography of flavonoids and saponins in *Gynostemma pentaphyllum* and their antiproliferation effect on hepatoma cell. *Phytomedicine* **2010**, *18*, 2–10.

(2) Tsai, Y. C.; Wu, W. B.; Chen, B. H. Preparation of carotenoids and chlorophylls from *Gynostemma pentaphyllum* Makino and their antiproliferation effect on hepatoma cell. *J. Med. Food* **2010**, *13*, 1431–1442.

(3) Schild, L.; Chen, B. H.; Makarov, P.; Kattengell, K.; Heinitz, K.; Keilhoff, G. Selective induction of apoptosis in glioma tumor cells by *Gynostemma pentaphyllum* extract. *Phytomedicine* **2010**, *17*, 589–597.

(4) Kim, Y. J.; Kwon, H. C.; Ko, H.; Park, J. H.; Kim, H. Y.; Yoo, J. H.; Yang, H. O. Anti-tumor activity of the ginsenoside Rk1 in human hepatocellular carcinoma cells through inhibition of telomerase activity and induction of apoptosis. *Biol. Pharm. Bull.* **2008**, *31*, 826–830.

(5) Wang, W.; Rayburn, E. R.; Hao, M.; Zhao, Y.; Hill, D. L.; Zhang, R.; Wang, H. Experimental therapy of prostate cancer with novel natural product anti-cancer ginsenosides. *Prostate* **2008**, *68*, 809–819.

(6) Chen, J. C.; Lu, K. W.; Tsai, M. L.; Hsu, S. C.; Kuo, C. L.; Yang, J. S.; Hsia, T. C.; Yu, C. S.; Chou, S. T.; Kao, M. C.; Chung, J. G.; Gibson, W. W. Gypenosides induced G0/G1 arrest via CHK2 and apoptosis through endoplasmic reticulum stress and mitochondria-dependent pathways in human tongue cancer SCC-4 cells. *Oral Oncol.* **2009**, *45*, 243–273.

(7) Circosta, C.; De Pasquale, R.; Occhiuto, F. Cardiovascular effects of the aqueous extract of *Gynostemma pentaphyllum* Makino. *Phytomedicine* **2005**, *12*, 638–643.

(8) Megalli, S.; Davies, N. M.; Roufogalis, B. D. Anti-hyperlipidemic and hypoglycemic effects of *Gynostemma pentaphyllum* in the Zucker fatty rat. *J. Pharm. Pharm. Sci.* **2006**, *9*, 281–291.

(9) Lee, H. U.; Bae, E. A.; Han, M. J.; Kim, N. J.; Kim, D. H. Hepatoprotective effect of ginsenoside Rb1 and compound K on tert-butyl hydroperoxide-induced liver injury. *Liver Int.* **2005**, *25*, 1069–1073.

(10) Jin, Y; Xiao, Y. S.; Zhang, F. F.; Xue, X. Y.; Xu, Q.; Liang, X. M. Systematic screening and characterization of flavonoid glycosides in Carthamus tinctorius L. by liquid chromatography/ UV diode-array detection/electrospray ionization tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2008**, *46*, 418–430.

(11) Sakata, K.; Hirose, Y.; Qiao, Z.; Tanaka, T.; Mori, H. Inhibition of inducible isoforms of cyclooxygenase and nitric oxide synthase by flavonoid hesperidin in mouse macrophage cell line. *Cancer Lett.* **2003**, *199*, 139–145.

(12) Chi, Y. S.; Lim, H.; Park, H.; Kim, H. P. Effects of wogonin, a plant flavone from *Scutellaria radix*, on skin inflammation: *In vivo* regulation of inflammation-associated gene expression. *Biochem. Pharmacol.* **2003**, *66*, 1271–1278.

(13) Tripoli, E.; Guardia, M. L.; Giammanco, S.; Majo, D. D.; Giammanco, M. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chem.* **2007**, *104*, 466–479.

(14) Kao, T. H.; Huang, S. C.; Inbaraj, B. S.; Chen, B. H. Determination of flavonoids and saponins in *Gynostemma pentephyllum* (Thunb.) Makino by liquid chromatography-mass spectrometry. *Anal. Chim. Acta* **2008**, *626*, 200–211.

(15) SPSS Statistics (SPSS). *SPSS Statistics Base User's Guide*, Version 17.0; SPSS Statistics for Microsoft Windows; SPSS Inc.: Chicago, IL, 2009.

(16) Borutaite, V. Mitochondria as decision-makers in cell death. *Environ. Mol. Mutagen.* **2010**, *51*, 406–416.

(17) Wang, Q. F.; Chen, J. C.; Hsieh, S. J.; Cheng, C. C.; Hsu, S. L. Regulation of Bcl-2 family molecules and activation of caspase cascade involved in gypenosides-induced apoptosis in human hepatoma cells. *Cancer Lett.* **2002**, *183*, 169–178.

(18) Lu, H. F.; Chen, Y. S.; Yang, J. S.; Chen, J. C.; Lu, K. W.; Chiu, T. H.; Liu, K. C.; Yeh, C. C.; Chen, G. W.; Lin, H. J.; Chung, J. G. Gypenosides induced G0/G1 arrest via inhibition of cyclin E and induction of apoptosis via activation of caspases-3 and -9 in human lung cancer A-549 cells. *In Vivo* **2008**, *22*, 215–221.

(19) Wang, C. Z.; Xie, J. T.; Fishbein, A.; Aung, H. H.; He, H.; Mehendale, S. R.; He, T. C.; Du, W.; Yuan, C. S. Antiproliferative effects of different plant parts of *Panax notoginseng* on SW480 human colorectal cancer cells. *Phytother. Res.* **2009**, *23*, 6–13.

(20) Zhang, Q.; Zhao, X. H.; Wang, Z. J. Flavones and flavonols exert cytotoxic effects on a human oesophageal adenocarcinoma cell line(OE33)

by causing G2/M arrest and inducing apoptosis. *Food Chem. Toxicol.* **2008**, *46*, 2042–2053.

(21) Zhang, Q.; Zhao, X. H.; Wang, Z. J. Cytotoxicity of flavones and flavonols to a human esophageal squamous cell carcinoma cell line (KYSE-510) by induction of G2/M arrest and apoptosis. *Toxicol. in Vitro* **2009**, *23*, 797–807.

(22) Xu, W.; Liu, J.; Li, C.; Wu, H. Z.; Liu, Y. W. Kaempferol-7-O-β-D- glucoside (KG) isolated from *Smilax china* L. rhizome induces G2/M phase arrest and apoptosis on HeLa cells in a p53-independent manner. *Cancer Lett.* **2008**, *264*, 229–240.

(23) Wang, B. F.; Wang, J. S.; Lu, J. F.; Kao, T. H.; Chen, B. H. Antiproliferation effect and mechanism of prostate cancer cell lines as affected by isoflavones from soybean cake. *J. Agric. Food Chem.* **2009**, *57*, 2221–2232.

(24) Shen, S. C.; Chen, Y. C.; Hsu, F. L.; Lee, W. R. Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase-3 cascade. *J. Cell Biochem.* **2003**, *89*, 1044–1055.

(25) Manthey, J. A.; Guthrie, N. Antiproliferative activities of citrus flavonoids against six human cancer cell lines. *J. Agric. Food Chem.* **2002**, *50*, 5837–5843.

(26) Ramos, S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *J. Nutr. Biochem.* **2007**, *18*, 427–442.

(27) Nguyen, T. T. T.; Tran, E.; Nguyen, T. H.; Do, P. T.; Huynh, T.; Huynh, H. The role of activated MEK−ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis* **2004**, *25*, 647–659.

(28) Kim, Y. H.; Lee, D. H.; Jeong, J. H.; Guo, Z. S.; Lee, Y. J. Quercetin augments TRAIL-induced apoptotic death: Involvement of the ERK signal transduction pathway. *Biochem. Pharmacol.* **2008**, *75*, 1946–1958.

(29) Galderisi, U.; Jori, F. P.; Giordano, A. Cell cycle regulation and neural differentiation. *Oncogene* **2003**, *22*, 5208–5219.

(30) Leonardi, E; Girlando, S; Serio, G; Mauri, F. A.; Perrone, G; Scampini, S; Dalla Palma, P Barbareschi M. PCNA and Ki67 expression in breast carcinoma: Correlations with clinical and biological variables. *J. Clin. Pathol.* **1992**, *45*, 416–419.