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Hepatoprotective Activity of Fresh *Polygonum Multiflorum* Against HEP G2 Hepatocarcinoma Cell Proliferation

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ABSTRACT

The modulatory activity of water extracts from fresh roots of *Polygonum multiflorum* (WRP) on oxidative stress in two liver cell lines was investigated for the first time. WRP increased antioxidant enzyme activities in Clone 9 cells, including catalase (CAT), glutathione-S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx). The intracellular glutathione (GSH) level was also increased in the Clone 9 cells owing to antioxidase activity elevating effect by WRP treatment for 24 h. On the other hand, reactive oxygen species (ROS) were generated in abundance upon WRP treatment in Hep G2 cells, resulting in the suppression of cell proliferation. Apoptosis was increased to 78% and 77% after WRP (1000 μg/mL) treatment for 24 and 48 h, respectively. Moreover, WRP stimulated the activation of caspase-8, caspase-9, and ultimately caspase-3. Hep G2 cell growth suppression and caspase induction by WRP were greater than that by emodin-like substances (WRPE), a major active compound. These results indicated that phytochemicals present in extracts of *P. multiflorum* root act synergistically to promote apoptosis, suggesting the possible use of these extracts for hepatoprotection.

Key words: *Polygonum multiflorum*, antioxidant enzyme, growth suppression, apoptosis, hepatoprotection

INTRODUCTION

Polygonum multiflorum, also known as he-so-wow, is an herb used in traditional Chinese folk medicine for treating coughs, influenza and nephritis[1]. *P. multiflorum* has been reported to contain several beneficial substances, including emodin, rhein, 2,3,5,4-tetrahydroxystilbene-2-O-beta-glucoside, physcion, chrysophanol and resveratrol[2,3]. Its principle active compounds[4] have demonstrated emodin-like anti-tumor, anti-viral, immune-regulating and anti-inflammatory activities[5,6].

Earlier studies demonstrated that emodin exerts greater pro-oxidative than anti-oxidative effects[7]. Emodin induces apoptosis of HL-60 cells *in vitro* through the generation of reactive oxygen species (ROS)[8], and causes cell cycle arrest at G2/M in the common hepatocellular carcinoma (HCC) cell line Hep G2/C3A through the activation of p53, p21, Fas/APO-1 and caspase-3 protein[9].

Different caspases mediate the two apoptotic signaling pathways. Initiation of the Fas signaling pathway by either Fas/death receptors or TNF-α receptors causes recruitment of Fas-associated protein with death domain (FADD) via interactions between the death domains of Fas and FADD. Caspase-8 is activated by the binding of procaspase-8 to the Fas/FADD complex, in turn activating caspase-3 and inducing apoptosis. The second apoptotic pathway is regulated by mitochondrial release of cytochrome c, resulting in activation of caspase-9 and caspase-3.

Phytochemicals arrest the cell cycle in cancer cells both by altering signal transduction pathways and by induction of apoptosis through the generation of ROS, and ensuing cell death is accompanied by activation of certain stress kinases[10]. One study indicates that GSH-S-transferase (GST) controls ROS generation and apoptosis by blocking MAP kinase kinase kinase (MAPKKK) and p38 MAP kinase[11], thereby protecting cells from upregulated expression of proapoptotic proteins and from growth inhibition[12,13].

P. multiflorum contains an abundance of emodin-like substances, and our previous high performance liquid chromatography (HPLC) study found 6.6 μg of emodin-like compounds per mg of WRP[14]. In the present study, we tested the effects of water-based extracts of fresh roots, stems and leaves of *P. multiflorum* on Clone 9 liver cell antioxidant activities. We also investigated the influence of WRP on cell growth, cell cycle, and apoptosis induction in Hep G2 hepatocarcinoma cells.

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MATERIALS AND METHODS

I. *Materials and Chemicals*

The root, stem and leaf of fresh *Polygonum multiflorum* were obtained from the arbor farm (Tainan, Taiwan). Emodin, Triton X-100, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), propidium iodide (PI) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) were obtained from E. Merck Co. Ltd. (Darmstadt, Germany). Annexin V-FITC apoptosis detection kit, caspase-3, -8 and -9 assay kits were obtained from Biovision (California, USA).

II. *Preparation of Water Extracts*

The root, stem and leaf (100 g) of fresh *P. multiflorum* were cut into 4-mm thickness and freeze-dried before water extraction (2 L) via supersonication at 37°C for 50 min, respectively. The solutions were filtered by filter paper, freeze-dried again and stored at -20°C prior to analysis. The root, stem and leaf extracts of fresh *P. multiflorum* were abbreviated as WRP, WSP and WLP, respectively.

III. *Cell Culture*

Clone 9 cell is a normal liver cell of rat (BCRC 60201) cultured in Ham's F-12K medium, and a hepatocellular carcinoma cell line, named Hep G2 cell (BCRC 60025) cultured in minimum essential medium (MEM, Eagle). Both media were respectively supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1.0 mM sodium pyruvate and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

IV. *Cell Viability*

Hep G2 and Clone 9 cells (1×10^5 cells/per well) were seeded into 48-well plates in 1 mL of MEM or Ham's F-12K medium overnight. The cells were treated with different concentration of samples. At the end of incubation, the cells were washed with PBS, and the supernatants were exchanged with 1 mL of medium and MTT (0.5 mg/mL) to react for 2 h at 37°C. After washing with PBS, the MTT tetrazolium was dissolved with DMSO and measured at 570 nm.

V. *Assay for Intracellular Antioxidase Activity*

Glutathione peroxidase (GPx) activity determination was described previously[15]. Briefly, 0.1 mL of cell homogenate was mixed with 0.8 mL of 100 mM potassium phosphate buffer (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/mL GR, and 1 mM GSH, pH 7.0) and incubated for 5 min at room temperature. Thereafter, the reaction was initiated after adding 0.1 mL of 2.5 mM hydrogen peroxide (H_2O_2). GPx activity was evaluated by calculating the change of the absorbance at 340 nm for 5 min. In another reaction, 0.1 mL of 0.1 M phosphate buffer (1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH, pH 7.0) was added to cell homogenate and glutathione reductase (GR) activity was determined by measuring the decreasing absorbance at 340 nm for 3 min[16]. The catalase (CAT) activity was determined according to the method of Aebi[17]. Fifty microliters of cell homogenate were mixed with 950 μL of 0.02 M H_2O_2 and incubated at room temperature for 2 min. CAT activity was calculated by recording the change in the absorbance at 240 nm for 3 min.

VI. *Annexin V/PI Double Staining Assay*

Cells were stained with annexin V-FITC/propidium iodide (PI) stain solution to analyze apoptotic and necrotic cells by FACScan flow cytometer (Becton-Dickinson Lmmunocytometery Systems USA, San Jose, CA). In brief, the cells were treated with various concentrations of different extracts for 24 and 48 h. The cells were then washed with PBS and suspended in 500 μL of binding buffer, and centrifuged to remove buffer. Subsequently, the cells were mixed with 5 μL of PI and 5 μL of annexin V-FITC to incubate in the dark for 10 min. The cells were then analyzed by FACScan flow cytometer. The CellQuest and WinMDI 2.8 software was used to analyze the apoptotic and necrotic cells.

VII. *Assay for ROS Level*

Hep G2 cells (2×10^6 cells/per well) were treated with various concentrations of different extracts for 24 h. Collected cells were suspended in 500 μL of PBS, and mixed with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to incubate for 20 min at 37°C. The cells were washed thrice with PBS to remove redundant DCFH-DA. The cell pellet was mixed with 500 μL of PBS and the ROS level was assayed by FACScan flow cytometer (Becton-Dickinson Lmmunocytometery Systems USA, San Jose, CA).

VIII. *Determination of Caspase-3,-8, and -9*

Caspase-3,-8, and -9 were determined with kits from Biovision (Mountain View, CA, USA). Cells (5×10^6 cells/per well) were treated with different extracts for 24 and 48 h. The cells were then washed with PBS and suspended in 50 μL of cell lysis buffer to incubate on ice for 10 min. Centrifugation (10,000 ×g) was performed for 1 min, and the supernatant (cytosolic extract) was transferred to fresh tubes and put on ice for immediate assay. Fifty microliters of 2X reaction buffer (containing 10 mM

DTT) added with 5 μL of the 4 mM substrate was added to the cytosolic extract, incubated at 37°C for 1 h and its absorbance at 405 nm was measured.

IX. *Statistical Analysis*

The above data were expressed as means ± SD. The software of ANOVA was used to evaluate the difference among multiple groups. If significant difference ($p < 0.05$) was observed, Duncan's multiple range test was used to compare the means of two specific groups.

RESULTS

I. *Effects of P. Multiflorum Extracts on Liver Cell (Clone 9 Cell Line) Viability and Antioxidant Enzyme Activities*

The yields of water extracts from root, leaf and stem of *P. multiflorum* were in descending order: root (25.1 g/100.0 g) > leaf (23.5 g/100.0 g) > stem (6.3 g/100.0 g). On the other hand, the antioxidant activities of plant or herb extracts are often explained with respect to their total phenolic and flavonoid contents. The total phenolic content of *P. multiflorum* root extract (179.0 ± 11.1 mg/g; WRP) was significantly higher than those of leaf extract (118.0 ± 6.0 mg/g; WLP) and stem extract (70.3 ± 1.4 mg/g; WSP) in our recent study[14]. As depicted in Figure 1, WRP was not cytotoxic toward Clone 9 liver cells at concentrations below 1000 μg/mL. We then tested WRP, WSP and WLP, each at 1000 μg/mL, for stimulation of intracellular antioxidant enzyme activities in Clone 9 cells (Table 1). All three plant extracts increased significantly CAT, GST, GR and GPx activities. WRP consistently produced the greatest stimulation thereby increasing the intracellular GSH, an important indicator for hepatoprotection.

II. *P. Multiflorum Root Extract Inhibits Hep G2 Hepatocarcinoma Cell Proliferation*

Our previous study showed the presence of 6.6 μg of emodin-like compounds (WRPE) per mg for WRP[14]. We therefore evaluated the cytotoxicity of WRPE against Hep G2 hepatocarcinoma cells by MTT assay. Figure 2 compares the dose-dependence of WRP on Hep G2 cell viability after a 24-h treatment. Both WRP (64.5%) and WRPE (60.1%) significantly reduced cell viability. Hence we further investigated the inhibitory activity for Hep G2 cell exposed to WRP or WRPE (Figure 3). Hep G2 cell proliferation was inhibited by 72% by WRP (1000 μg/mL) treatment for 48 h, whereas WRPE (6.6 μg/mL of emodin) inhibited growth by approximately 68%. Neither WSP nor WLP inhibited Hep G2 cell growth in 24-h treatments.

III. *Apoptotic and Necrotic Effects of P. Multiflorum Root Extracts*

Unrepaired DNA damage triggers signaling cascades leading to cell cycle arrest[18]. Exposure of Hep G2 cells to WRP at 1000 μg/mL led to cell cycle arrest at the G2/M

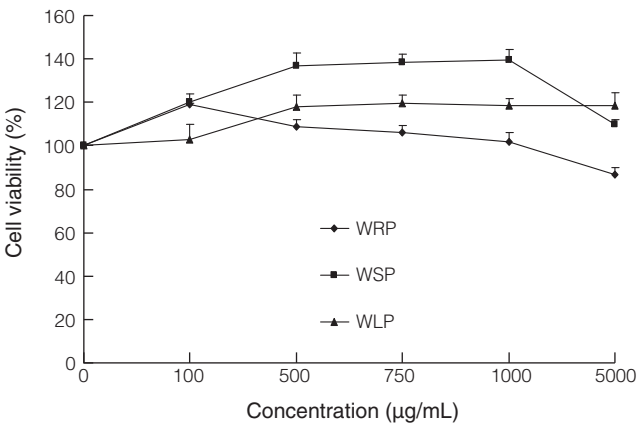

Figure 1. Cytotoxic effects of the water-based extracts from fresh *P. multiflorum* on Clone 9 cells. Each value was expressed as mean ± SD (n = 3). WRP: the water-based extracts from the root of fresh *P. multiflorum*; WSP: the water-based extracts from the stem of fresh *P. multiflorum*; WLP: the water-based extracts from the leaf of fresh *P. multiflorum*. Clone 9 cells were treated with WRP, WSP and WLP for 24 h.

Table 1. The promotion effect of fresh *Polygonum multiflorum* on antioxidase activities of Clone 9 cells

Group	Antioxidase (nmoles/min/mg protein)				Antioxidase (μmole/mg protein)
	GPx	GR	GST	CAT	GSH
Control	115 ± 33^c	7.3 ± 2.4^b	356 ± 18^c	11.5 ± 2.0^c	67 ± 6^b
WRP (1000 μg/mL)	237 ± 37^a	13.3 ± 3.9^a	738 ± 68^a	20.3 ± 2.4^a	142 ± 7^a
WSP (1000 μg/mL)	168 ± 38^b	6.9 ± 2.4^b	413 ± 42^b	12.2 ± 0.7^c	68 ± 4^b
WLP (1000 μg/mL)	228 ± 32^a	12.0 ± 1.7^a	463 ± 91^b	15.6 ± 2.3^b	75 ± 9^b

Each value was expressed as mean ± SD (n = 3). WRP: the water-based extracts from the roots of fresh *P. multiflorum*; WSP: the water-based extracts from the stem of fresh *P. multiflorum*; WLP: the water-based extracts from the leaf of fresh *P. multiflorum*. Clone 9 cells were respectively treated with WRP, WSP and WLP for 24 h. Values with different superscript letters are statistically different ($p < 0.05$) in each column.

boundary, and also significantly increased sub G1 phase (Table 2). Subsequently, the apoptosis induction of WRP was evaluated by FITC-conjugated annexin V and propidium iodide (PI) stain. When cells are stained with annexin V-FITC and PI, four different cell populations may be observed: (1) live cells that do not stain with either annexin V-FITC or PI; (2) necrotic cells that stain with PI; (3) apoptotic cells that stain with annexin V-FITC only (early apoptosis); and (4) apoptotic cells that stain both with PI and annexin V-FITC (late apoptosis). The apoptotic ratios were 78% and 77%, following treatment with WRP (1000 μg/mL) for 24 and 48 h, respectively. In contrast, after 24 and 48-h treatment with WRPE (6.6 μg/mL), the apoptotic ratios were only 46 and 65%, respectively. Thus, WRP demonstrated greater inhibitory capacity than WRPE (Table 3).

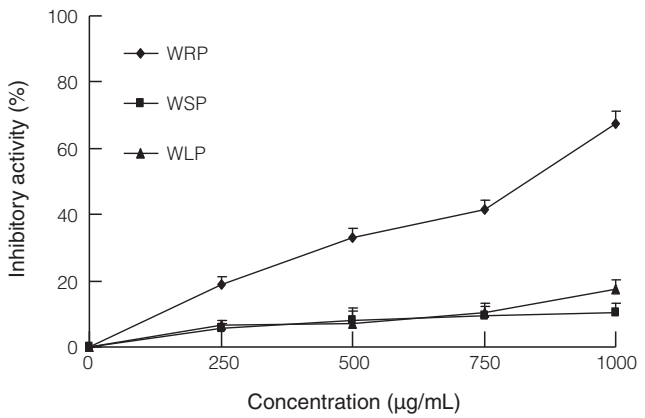

Figure 2. Cytotoxic effects of WRP, WSP, and WLP on Hep G2 cells for 24 h. Each value was expressed as mean ± SD (n = 3). Our previous study showed the presence of 6.6 μg of emodin-like compounds (WRPE) per mg of WRP. The inhibitory activity of WRPE1000 (6.6 μg/mL of emodin) was 60.1%.

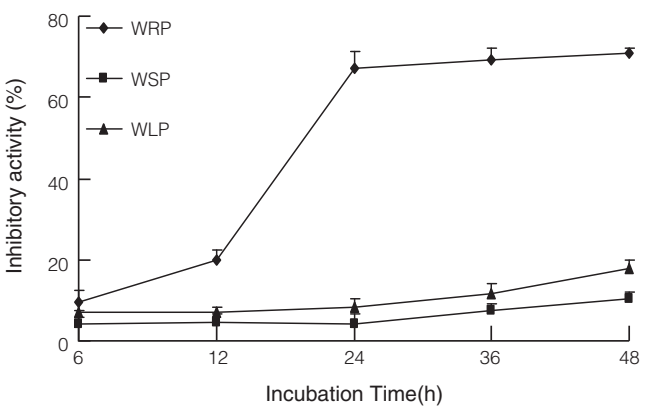

Figure 3. Cytotoxic effects of 1000 μg/mL WRP, WSP, and WLP on Hep G2 cells from 6 to 48 h. Each value was expressed as mean ± SD (n = 3). Our previous study showed the presence of 6.6 μg of emodin-like compounds (WRPE) per mg of WRP. The inhibitory activity of WRPE1000 (6.6 μg/mL of emodin) was 68%.

IV. *Effects of P. Multiflorum Root Extracts on ROS Production*

Phytochemicals have been shown to influence ROS-mediated processes including cell cycle arrest, apoptosis, differentiation and proliferation[19]. We employed 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to measure intracellular oxidant production in Hep G2 cells. DCFH-DA is a non-fluorescent cell-permeant hydrophobic compound which undergoes cleavage by intracellular esterases to form hydrophilic and cell-impermeant

Table 2. The cell cycle of Hep G2 cells treated with fresh *Polygonum multiflorum* extracts

Group	SubG1 (%)	Non-apoptotic cells (%)		
		G0/G1	S	G2/M
Control	1.9 ± 0.8	52.8 ± 0.6	25.2 ± 1.0	20.1 ± 2.5^{b}
WRP100	1.6 ± 0.5	49.6 ± 4.7	21.2 ± 7.5	27.6 ± 4.9^{b}
WRP1000	8.3 ± 2.0	27.6 ± 7.1	25.2 ± 2.0	38.9 ± 0.9^{a}
WRPE100	1.1 ± 0.5	48.0 ± 3.0	25.5 ± 1.2	25.4 ± 2.0^{b}
WRPE1000	0.6 ± 0.5	49.9 ± 3.4	23.8 ± 0.5	25.7 ± 1.1^{b}

Each value was expressed as mean ± SD (n = 3). Our previous study showed the presence of 6.6 μg of emodin-like compounds (WRPE) per mg of WRP. WRP100: the water-based extracts from the root of fresh *P. multiflorum* at 100 μg/mL; WRPE100 (0.66 μg/mL of emodin): the dosage of emodin was equivalent to emodin-like compounds in 100 μg/mL WRP; WRP1000: the water-based extracts from the root of fresh *P. multiflorum* at 1000 μg/mL; WRPE1000 (6.6 μg/mL of emodin): the dosage of emodin was equivalent to emodin-like compounds in 1000 μg/mL WRP. Hep G2 cells were respectively treated with WRP and WRPE for 24 h.

Table 3. The effects of WRP and WRPE-induced apoptosis on Hep G2 cells

	Groups	Percentage (%)			
		Normal	Early apoptosis	Late apoptosis	Necrosis
24 h	Control	80.9 ± 0.3^{a}	16.4 ± 0.1^{c}	1.7 ± 0.5^{c}	1.0 ± 0.5^{b}
	WRP1000	19.8 ± 0.5^{c}	70.5 ± 0.8^{a}	7.7 ± 0.2^{a}	2.0 ± 0.4^{b}
	WRPE1000	50.3 ± 0.4^{b}	40.7 ± 0.5^{b}	5.3 ± 0.3^{b}	3.7 ± 0.3^{a}
48 h	Control	85.7 ± 0.3^{a}	11.4 ± 0.6^{c}	1.7 ± 0.2^{b}	1.2 ± 0.2^{b}
	WRP1000	22.5 ± 0.4^{c}	75.0 ± 0.5^{a}	2.4 ± 0.1^{b}	0.1 ± 0.1^{c}
	WRPE1000	32.4 ± 0.3^{b}	60.5 ± 0.5^{b}	4.7 ± 0.4^{a}	2.5 ± 0.1^{a}

Each value was expressed as mean ± SD (n = 3). Our previous study showed the presence of 6.6 μg of emodin-like compounds (WRPE) per mg of WRP. WRP1000: the water-based extracts from the root of fresh *P. multiflorum* at 1000 μg/mL; WRPE 1000 (6.6 μg/mL of emodin): the dosage of emodin was equivalent to emodin-like compounds in 1000 μg/mL WRP. Values in each column with different superscript letters are in significant difference ($p < 0.05$).

DCFH. Oxidation of DCFH produces the fluorescent species, dichlorofluorescein, so that this probe serves as a reporter of intracellular ROS. In Hep G2 cells loaded with the probe, WRP distinctly increased ROS production (Figure 4). After treatment for 24 h, the relative fluorescence values (% of control) of cells treated with WRP at 100 and 1000 μg/mL were 120 and 155%, respectively. These results demonstrated that WRP stimulated ROS production in a concentration-dependent manner. In contrast, this pro-oxidative effect was not observed in cells treating with WRPE (0.66 or 6.6 μg/mL).

V. *Activation of Caspase-3, -8, and -9*

Activation of apoptosis through either the caspase-9-mediated mitochondrial pathway or the caspase-8-mediated death receptor pathway leads to the activation of caspase-3. The caspase-3, -8, and -9 activities were measured in Hep G2 cells treating with 1000 μg/mL WRP (WRP1000) or 6.6 μg/mL WRPE (WRPE1000) for 24 h (Figure 5). The results demonstrated that caspase-9 activity was increased both in the WRP1000 (200%) and WRPE (175.9%) comparing to the control group (100%) after 24 h induction. Furthermore, caspase-3 and caspase-8 activities both were increased by WRP1000 (197.0% and 174.4%) and WRPE (193.9% and 176.7%) treatments. However, the stimulatory activity for caspase-9 by WRP1000 was greater than WRPE1000.

DISCUSSION

The liver is highly sensitive to oxidative stress, defined as an imbalance between anti-oxidative defenses and ROS levels[20]. Excessive free radicals cause damage to bioactive molecules, including lipids, proteins and DNA, resulting in inactivation of antioxidant enzymes, and ultimately, liver cell death. Many phytochemicals offer significant protective benefits against oxidative damage[19]. While ethanol extracts of *P. multiflorum* roots

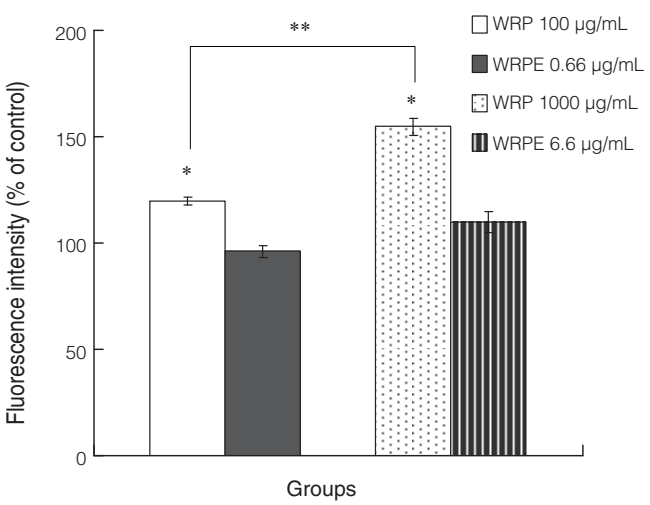

Figure 4. The ROS production levels of Hep G2 cells with WRP and WRPE treatment. Each value was expressed as mean ± SD (n = 3). Our previous study showed the presence of 6.6 μg of emodin-like compounds (WRPE) per mg of WRP. Hep G2 cells were separately treated with WRP and WRPE for 24 h. *WRP *vs.* WRPE ($p < 0.05$). **WRP100 *vs.* WRP1000 ($p < 0.05$).

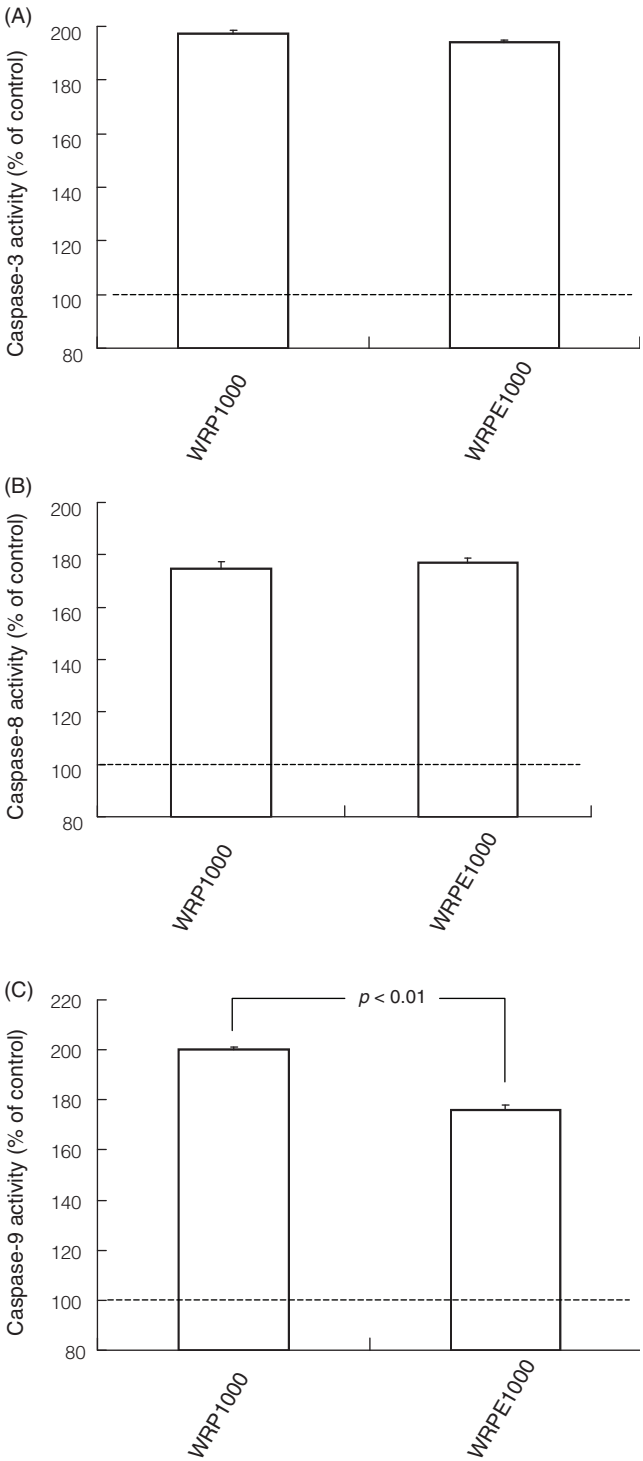

Figure 5. The caspase-3 (A), caspase-8 (B), and caspase-9 (C) activities (% of control) in the Hep G2 cells by WRP and WRPE treatment. The activity was as 100% in the control group. Each value was expressed as mean ± SD (n = 3). WRP1000 *vs.* WRPE1000 ($p < 0.01$).

were found to stimulate antioxidant activities in liver, heart and brain of rats[21,22], and WRP was shown to contain significant antioxidant capacity *in vitro*[14], the hepatoprotective properties of fresh *P. multiflorum* extracts had not been investigated previously. In this study we have shown that WRP stimulates liver cell antioxidant activities, suggesting that root extracts may represent a potent source of phytochemicals for liver chemoprevention.

In HL-60 cells, emodin causes ROS generation and caspase-3 activation[8], increases cleavage of poly(ADP-ribose) polymerase to inhibit DNA synthesis[23], and arrests the cell cycle[24]. The experimental results demonstrated that WRP significantly promotes Hep G2 cell apoptosis, and its proapoptotic capacity is greater than that of WRPE (Table 3). Treatment of Hep G2 cells with WRP (1000 μg/mL) for 24 h led to the activation of caspase-9, and to a lesser extent caspase-8 (Figure 5), indicating the engagement of both the mitochondrial and Fas/death receptor pathways. Moreover, the potency of WRP in activating caspase-3 was greater than that of WRPE, which would be consistent with WRP having greater antiproliferative effects on Hep G2 cells than WRPE.

The proapoptotic capacity of water extracts of *P. multiflorum* roots was even higher than that of emodin, and we believe that this extra proapoptotic potency may be due to synergism between emodin-like compounds and other phytochemicals present in the extracts. Indeed, WRP treatment significantly increased intracellular ROS production in Hep G2 cells, while WRPE did not. This study also demonstrated greater stimulation of intracellular antioxidant activities by the extract of the root of the plant than by the extracts of its stem or leaf. We propose that *P. multiflorum* roots might be a valuable source of useful chemotherapeutic agents to stimulate antioxidant activities in normal liver cells while inducing apoptosis in cancer cells.

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