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Flavonoid Fraction Purified from *Rhus verniciflua* Stokes Actively Inhibits Cell Growth Via Induction of Apoptosis in Mouse Tumorigenic Hepatocytes

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Abstract – Dietary flavonoids are currently receiving considerable attention in developing novel cancer-preventive approaches because of their potential capacities to actively induce apoptosis of cancer cells. In our previous report, a flavonoid fraction, which consisted mainly of protocatechuic acid, fustin, fisetin, sulfuretin, and butein and named RCMF (RVS chloroform-methanol fraction), was prepared from a crude acetone extract of *Rhus verniciflua* Stokes (RVS) that is traditionally used as food additive and herbal medicine. In this study, we evaluated the effects of the RCMF on cell proliferation and apoptosis using SV40-transformed tumorigenic hepatocytes, BNL SV A.8. Tritium uptake assay showing the proliferative capacity of the cells was strongly suppressed in the presence of RCMF. This anti-proliferative effect was further confirmed through trypan blue exclusion. RCMF-mediated suppression of cell growth was verified to be apoptotic, based on the increase in DNA fragmentation, low fluorescence intensity in nuclei after propidium iodide staining, and the appearance of DNA laddering. Collectively, this study demonstrated that RCMF can be approached as a potential agent that is capable of significantly inhibiting cell growth of hepatic cancer cells.

Keywords – *Rhus verniciflua* Stokes, Flavonoids, Hepatocytes, Growth inhibition, Apoptosis

Introduction

In recent years, there has been a global trend toward the use of natural bioactive substances as cancer chemopreventive or therapeutic agents (Pezutto, 1997; Christou *et al.*, 2001; Mukherjee *et al.*, 2001). Most of these substances exert their chemotherapeutic activity by blocking cell cycle progression and triggering apoptotic cell death. Therefore, induction of apoptosis in tumor cells has become an important indicator of the tumor treatment response in employing a plant derived-bioactive substance to control human mortality due to cancer (Smets, 1994; Paschka *et al.*, 1998).

Rhus verniciflua Stokes (RVS) has long been used as food additive and herbal medicine (Jung, 1998; Hong *et al.*, 1999). Recently, various biological activities of RVS have been reported such that RVS contained antioxidant and antitumor activities (Lee *et al.*, 1999; Kitts and Lim, 2001; Lee *et al.*, 2001). Previously, we prepared a purified RVS extract and demonstrated the antioxidant and antitumor potentials of the extract, suggesting that flavonoids are the major active compounds responsible for the biological activity of RVS (Lee *et al.*, 2002; Lee *et al.*, 2003). Flavonoids are commonly found in most plants and exert a remarkable spectrum of biological activities affecting basic cell functions, such as proliferation, differentiation, and apoptosis (Formica and Regelson, 1995; Plaumann *et al.*, 1996; Caltagirone *et al.*, 2000). Furthermore, flavonoids are dietary pharmacological agents that might block the neoplastic inception or delay disease progression (Gao *et al.*, 1999; Wong and McLean, 1999). Collectively, we hypothesized that flavonoids contained in RVS are capable of inducing growth inhibition and apoptosis of cancer cells. In order to determine this hypothesis, we prepared a purified flavonoid fraction from a crude acetone extract of RVS and evaluated the effects of the RVS fraction in inhibiting cell growth and inducing apoptosis in mouse tumorigenic hepatocytes.

Materials and Methods

Chemicals and laboratory wares – Unless otherwise specified, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) and all the laboratory wares were from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ).

Preparation of RVS sample – A purified flavonoid fraction was prepared from a crude acetone extract of RVS wood as described previously (Jung, 1998). Briefly,

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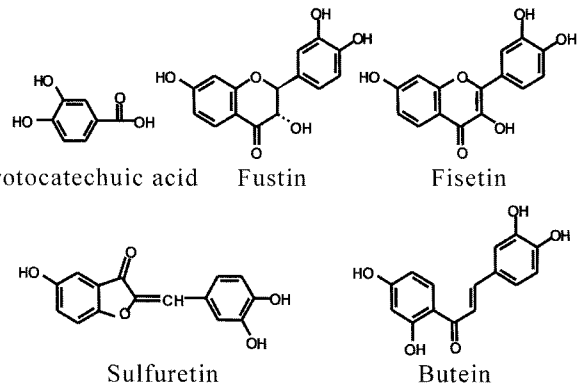

Fig. 1. Chemical structure of five compounds identified from RCMF.

five grams of the crude extract dissolved in methanol underwent silica gel adsorption column chromatography (2.9 x 45 cm, 230-400 mesh) and then eluted with 200 ml of organic solvent mixture [chloroform:methanol (90:10, v/v)]. The eluted solution was collected, evaporated to 3.7 g (74% of the initial amount), and named RCMF (RVS chloroform-methanol fraction). After RCMF was further purified by HPLC to identify its chemical composition, five prominent peaks were observed and the compounds contained within peaks 1 to 5 were identified as protocatechuic acid, fustin, fisetin, sulfuretin, and butein, respectively, by comparing the EI-MS and NMR spectra with that of reported previously (Jung, 1998; Zhang *et al.*, 1998; Kim *et al.*, 1999; Lee *et al.*, 2002) (Fig. 1). Especially, compound 1 exhibited an MS molecular ion peak at *m/z* 154 that corresponded to $C_7H_3O_4$, and its NMR spectra with δ 6.80 (1H, d, J = 8.0 Hz, H-5), 7.42 (1H, d, J = 2.0 Hz, H-6), 7.43 (1H, dd, J = 2.8, 8.0 Hz, H-2) for ^{1}H-NMR, and δ 114.77 (d, C-5), 116.75 (d, C-2), 122.90 (d, C-6), 122.19 (s, C-1), 145.06 (s, C-3), 150.52 (s, C-4), 169.21 (s, C-7) for ^{13}C-NMR indicated 3,4-dihydroxybenzoic acid, *i.e.*, protocatechuic acid. In addition, compound 3 was identified as fisetin, because its ^{1}H-NMR spectra showed a tetrahydroxyflavone signal with δ 6.91-6.93 (3H, m, H-6, 8, 5'), 7.68 (1H, dd, J = 2.0, 8.0 Hz, H-6'), 7.78 (1H, d, J = 2.0 Hz, H-2'), 8.00 (1H, d, J = 6.0 Hz, H-5') and its ^{13}C-NMR and EI-MS spectra were also consistent with previous data (Jung, 1998). These two compounds, protocatechuic acid and fisetin, were not presented in the previously reported HPLC profiles obtained with RVS analyses (Lee *et al.*, 2002). RCMF was dissolved freshly in dimethylsulfoxide (DMSO) and the final concentration of DMSO did not exceed 0.1% (v/v) throughout the experiments. Concentrations used in this experiment are expressed as the dry weight of RCMF (μg/ml).

Cell culture and treatment – SV40-transformed mouse tumorigenic hepatic cell line, BNL SV A.8, were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). Prior to RCMF treatment, cultures were switched to a fresh batch of the same medium. At various times after treatment, the cells were processed for the analyses of cell growth and apoptosis.

Measurement of DNA synthesis – The level of DNA synthesis by the hepatic cells after RCMF treatment was measured by adding 1 μCi of [methyl-^{3}H] Thymidine (Amersham Pharmacia Biotech Inc., Piscataway, NJ) to each well of 96-well culture plates for the last 12 h of various culture periods. The cells were then collected with a cell harvester (Inotech Inc., Switzerland), and the tritium contents were measured using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

Determination of cytotoxicity – Cellular cytotoxicity induced by RCMF treatment was measured using trypan blue exclusion assay. Briefly, the hepatic cells were cultured in DMEM supplemented with 10% FBS in the presence of 1 to 100 μg/ml RCMF for various times. After incubation, the cells were stained with 0.4% trypan blue and about 100 cells were counted for each treatment. Cytotoxicity was calculated as follows: % cytotoxicity = [(total cells - viable cells) / total cells]×100.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay – After exposure to RCMF for various times, the hepatic cells were fixed with 1% buffered formaldehyde (pH 7.5) for 30 min on ice. The cells were then washed with PBS, resuspended in 70% ice-cold ethanol, and kept at –20°C for 1 h. The cells were rehydrated with PBS and incubated in TdT buffer containing 30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM $CoCl_2$, 0.05 mg/ml BSA, 0.1 mM DTT, 7.5 U/ml TdT, and 0.4 nM/ml FITC-5-dUTP. After a 30-min incubation at 37°C, the reaction was blocked by transferring the cells to a buffer containing 300 mM sodium chloride, 30 mM sodium citrate, and 2% bovine serum albumin for 30 min. Finally, the cells were washed with PBS and observed under a fluorescence microscope (Axioskop 2, Carl Zeiss, Germany).

DNA fragmentation assay – After RCMF treatment, the hepatic cells were incubated with lysis buffer (1% NP-40 and 1% SDS in 50-mM Tris-HCl, pH 8.0) for 1 h at 65°C. DNA was extracted with phenol/chloroform/isoamyl alcohol and the degree of fragmentation was analyzed using 2% agarose gel electrophoresis followed by ethidium bromide staining.

Cell cycle analysis – RCMF-induced DNA fragmentation was also determined by flow cytometric analysis after propidium iodide (PI) staining. Initially, the suspension (2×10^6 cells) of RCMF-treated cells was fixed for 24 h at 4°C with 80%

ethanol and then incubated overnight at 4°C with 1 ml of PI staining mixture (250 μl of PBS, 250 μl of 1 mg/ml RNase in 1.12% sodium citrate, and 500 μl of 50 μg/ml PI in 1.12% sodium citrate). After the staining, 1×10^4 cells were analyzed with FACS Calibur® system (Becton Dickinson, San Jose, CA).

Statistical analyses – All the results are expressed as the mean ± standard error (SE). A one-way ANOVA using SPSS ver. 10.0 software was used for multiple comparisons. A value of $p < 0.05$ was considered significant.

Results

Effects of RCMF on proliferation of BNL SV A.8 cells – The effect of RCMF on cell proliferation was determined by the level of tritium incorporation using BNL SV A.8 cells. As shown in Fig. 2, the addition of RCMF to BNL SV A.8 cells dramatically inhibited DNA synthesis in a dose-dependent manner. When 10 μg/ml of RCMF was added to the cells, tritium uptake was 67,816 ± 5,482 cpm, representing a decrease of about 18.5% from the uptake in untreated cells (83,230±2,340 cpm). Treatment with 100 μg/ml of RCMF transparently inhibited tritium uptake by the hepatic cells, with an incorporation of only 35,324±3,800 cpm. In addition, RCMF-mediated inhibition of DNA synthesis in the cells was time dependent (Fig. 2B).

Effects of RCMF on cytotoxicity of BNL SV A.8 cells – Subsequently, we determined if RCMF exerted a cytotoxic effect on the hepatic cells by monitoring trypan blue exclusion after the RCMF treatment. The added RCMF exert a significant cytotoxic effect and the cytotoxicity induced by a 24 h incubation of BNL SV A.8 cells with 100 μg/ml RCMF was approximately 20% (Fig. 3A). A time-dependent increase in RCMF-mediated cytotoxicity

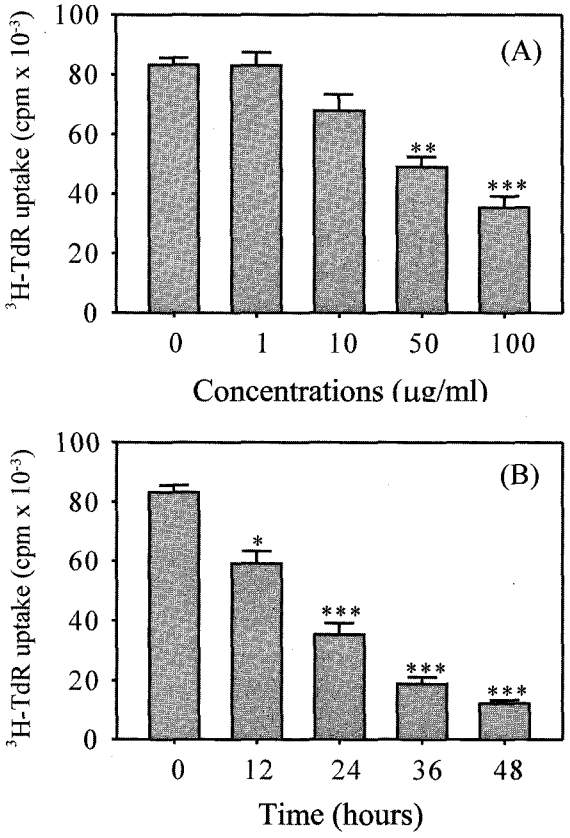

Fig. 2. Effect of RCMF on DNA synthesis in BNL SV A.8 cells. (A) The cells were treated with the indicated concentrations of RCMF for 24 h and then incubated for another 12 h in the presence of [methyl-^{3}H] TdR. (B) The cells were treated with 100 μg/ml RCMF for the indicated times and incubated with [methyl-^{3}H] TdR for the last 12 h of the incubation period. Each bar shows the mean ± SE of the experiments performed in triplicate. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ represent significant differences between the experimental and control value.

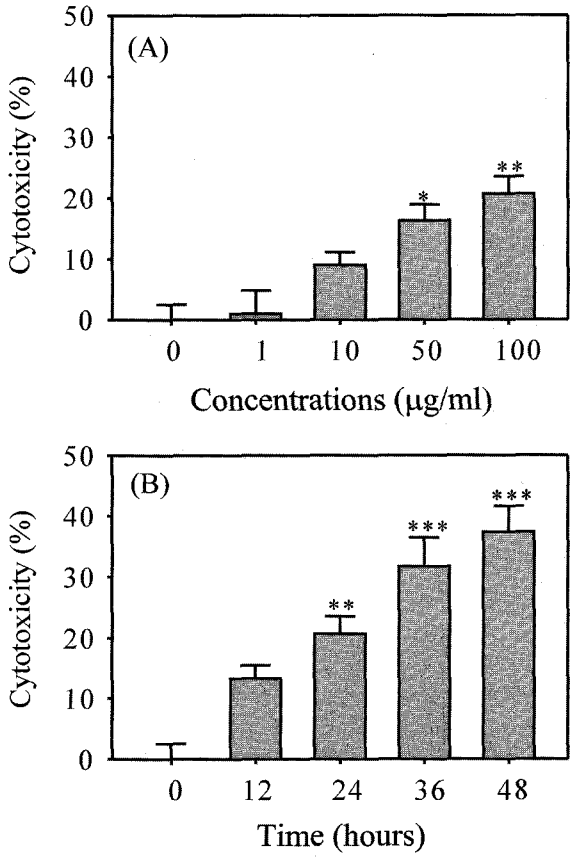

Fig. 3. Cytotoxic effects of RCMF in BNL SV A.8 cells. Cells were treated with the indicated concentrations of RCMF for 24 h (A) or with 100 μg/ml RCMF for the indicated times (B), and then processed for trypan blue staining. Each bar shows the mean ± SE of three separate experiments. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ represent significant differences between the experimental and control value.

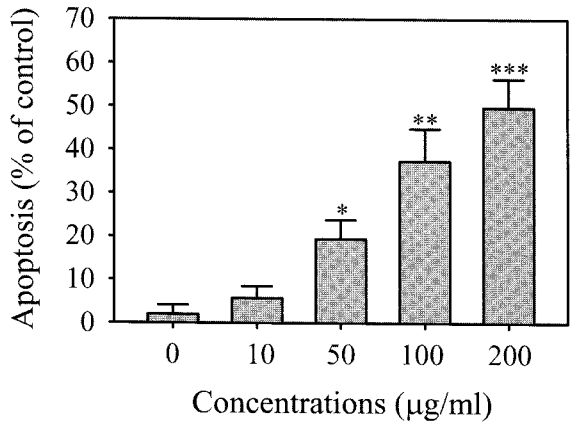

Fig. 4. TUNEL staining of BNL SV A.8 cells. Cells were treated with various concentrations (0-200 μg/ml) of RCMF for 24 h. After incubation, the cells were stained with FITC-conjugated dUTP and the degree of apoptosis was assessed. Each bar shows the mean ± SE of three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ represent significant differences between the experimental and control value.

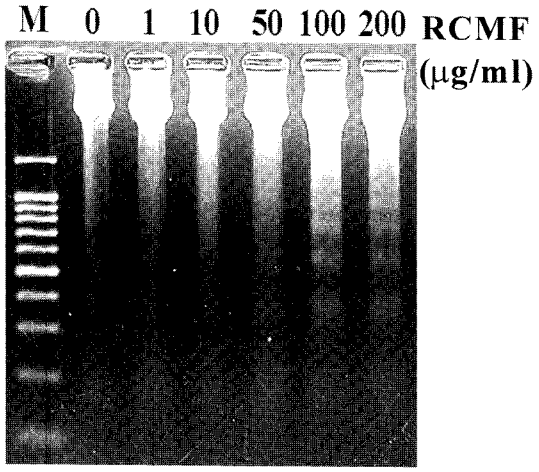

Fig. 5. Analysis of DNA fragmentation using agarose gel electrophoresis. BNL SV A.8 cells were incubated in the presence of 1 to 200 μg/ml of RCMF for 24 h. Genomic DNA was prepared and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. A representative result from three independent experiments is shown.

was also detected (Fig. 3B). Following a 48 h incubation with 100 μg/ml of RCMF in the cells, cytotoxicity was determined to be 37.3%.

RCMF-mediated apoptosis in BNL SV A.8 cells-To more understand the effect of RCMF on hepatic cells, RCMF-treated hepatocytes were subjected to apoptosis assays, including TUNEL assay, agarose gel electrophoresis of genomic DNA, and cell cycle analysis after PI staining (Figs. 4-6). Initially, a dose-dependent increase in the number of positively stained BNL SA A.8 cells was observed in the TUNEL assay after RCMF treatment (Fig. 4). For

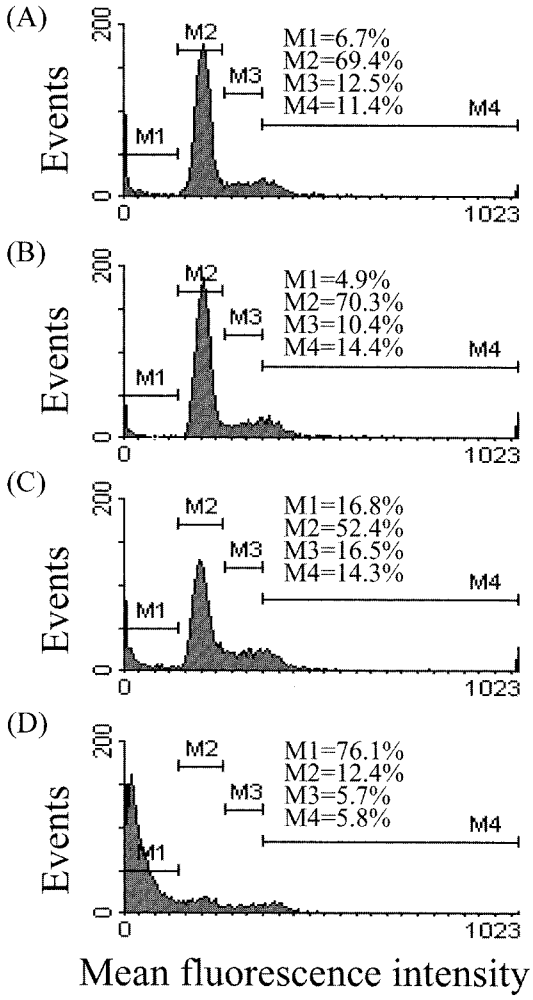

Fig. 6. Effect of RCMF on the cell cycle distribution of BNL SV A.8 cells. Cells were incubated in the absence (A) and presence of 10 (B), 50 (C), and 100 μg/ml of RCMF (D) for 48 h. The percentage of cells in each phase of the cell cycle was calculated through WinMDI 2.8 program of PI fluorescence data. A representative result from three independent experiments is shown. M1, M2, M3, and M4 indicate sub-G_0/G_1 (apoptotic) phase, G_0/G_1 phase, S phase, and G_2/M phase of cell cycle, respectively.

example, after 24 h of exposure to 100 or 200 μg/ml RCMF, apoptosis was seen in 37.3% and 49.7% of the cells, respectively. The induction of RCMF-mediated apoptosis was further examined by investigating DNA ladder formation after RCMF treatment (Fig. 5). As shown the figure, RCMF treatment dose-dependently induced apoptotic laddering of genomic DNA in the hepatic cells and the formation of DNA ladders was started from dose of 50 μg/ml. Finally, the induction of apoptosis in the cells by the RCMF treatment was confirmed with PI staining (Fig. 6). For example, following treatment with 50 or 100 μg/ml RCMF for 48 h, 16.8% and 76.1% of the cells became apoptotic, respectively.

Discussion

Current studies involved in developing cancer preventive approaches have been focused on identifying natural bioactive agents that are capable of actively inducing apoptosis of cancer cells (Mukherjee *et al.*, 2001). Flavonoids are present in most plants and known to exert various biological effects (Formica and Regelson, 1995; Plaumann *et al.*, 1996; Caltagirone *et al.*, 2000). Furthermore, flavonoids have antioxidant, vasodilatory, anticarcinogenic, anti-inflammatory, antibacterial, immune-stimulating, anti-allergic, and antiviral activities (Duarte *et al.*, 1993; Gao *et al.*, 1999; Wong and McLean, 1999). Therefore, flavonoids are believed to be an ideal agent for cancer chemoprevention.

Previously, we prepared a purified flavonoid sample from a crude acetone extract of RVS and named it RCMF. In this study, we evaluated the effects of RCMF on cell growth by determining the level of tritium incorporation in BNL SV A.8 cells. Results from the tritium uptake assay showed that the cells were very sensitive to RCMF-mediated inhibition of proliferation (Figs. 2A and 2B). In addition, the trypan blue staining experiment revealed that the viability of the cells was also significantly reduced by the RCMF treatment in a dose- and time-dependent manner (Figs. 3A and 3B). However, the RCMF treatment to mouse normal embryonic hepatocytes, BNL CL.2, did not induce any cytotoxicity at least under the same time and dose of the RCMF treatment (data not shown).

To understand the inhibition of cell growth by the RCMF in the cells, RCMF-treated BNL SV A.8 cells were subjected to apoptosis assays. As evidenced by the increased number of positively stained cells in the TUNEL assay (Fig. 4) and the induction of characteristic nuclear DNA ladders (Fig. 5), RCMF treatment actively induced apoptosis in the cells. The RCMF-mediated induction of apoptosis in the cells was further confirmed by the migration of many cells to sub-G1 phase (Fig. 6).

Among the promising strategies that can be approached as a novel cancer chemoprevention, a sensitive induction of apoptosis is regarded as one of the best ideal way to remove tumor cells. Since almost artificial agents currently used in cancer therapy are known to be toxic and produce severe damage to normal cells, naturally occurring agents that are capable of selectively eliminating cancer cells by inhibiting cell cycle progression and/or causing apoptosis are quite emphasized as promising agents for the therapy in recent years (Gupta *et al.*, 2001). Collectively, the results from apoptosis analyses suggested that RCMF could be used as a novel cancer chemopreventive agent. We assumed that the RCMF-induced apoptosis induction in the BNL SV A.8 cells is closely related to the inhibition of the activity of signal transduction molecules, since flavonoids have been reported to exert inhibitory effects on many kinds of protein kinases and some transcriptional factors (Yang *et al.*, 1998; Gamet-Payrastre *et al.*, 1999; Miranda *et al.*, 1999). In particular, several members of the mitogen-activated protein (MAP) kinase family, such as extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and p38 are reported to regulate the activation of AP-1 that play a critical role in tumor promotion (Whitmarsh and Davis, 1996). In contrast, flavonoids are known to modulate the activation of these MAP kinases and AP-1, although the pattern of regulation might differ depending on the chemical nature of the stimuli exposed and the type of cells being studied (Yoshioka *et al*, 1995; Lee *et al.*, 2003). Consequently, we believe that RCMF induced apoptosis of the hepatic cells via MAP kinase and/or AP-1-mediated signaling pathway. However, additional experiments should be performed to determine the precise mechanism(s) by which RCMF actively induces apoptosis in the mouse tumorigenic hepatic cells.

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