Activity of Crude Extract of *Rubus crataegifolius* Roots as a Potent Apoptosis Inducer and DNA Topoisomerase I Inhibitor

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The effects of methanol extract of *Rubus crategifolius* roots and its solvent fractions were investigated on the proliferation of MCF-7 human breast carcinoma cells. The methanol extract inhibited the proliferation of MCF-7 cells in a concentration dependent manner. Moreover, their methanol soluble (W-M) fraction had the greatest inhibitory effect on the growth of MCF-7 cells. To evaluate whether the W-M fraction affects on the cell cycle of MCF-7 cells, cells treated with this fraction were analyzed with flow cytometry. The W-M fraction increased G_0/G_1 phase after 24 h-treatment and induced apoptosis after 48 h-treatment. The hallmark of apoptosis, DNA fragmentation, also appeared by W-M fraction after 48 h-treatment. Furthermore, the methanol extract and its W-M fraction inhibited the activity of the topoisomerase I enzyme in the relaxation assay. From these results, their W-M fraction as well as methanol extract of *R. crategifolius* roots are necessary for further studies as a potent inhibitor of the growth of cancer cells.

Key words: *Rubus crategifolius* roots, MCF-7 cells, Topoisomerase I inhibitor, Cell cycle, Apoptosis, DNA fragmentation

INTRODUCTION

In Korea and China, some fruits of *Rubus* spp. have been used as a traditional medicine. In relation to biological activity of *R.* spp., radical scavenging effect of *R.* species (Constantino *et al.*, 1992) and antibacterial activity of *R. pinfaensis* (Richard *et al.*, 1994) have been reported. A number of terpenoids (Ganguly, 1972; Sarkar and Ganguly, 1978; Mukherjee *et al.*, 1984; Hattori *et al.*, 1988) and their glycosides from leaves (Zhou *et al.*, 1992; Ohtani *et al.*, 1992), roots (Gao *et al.*, 1985; Choi *et al.*, 1991), and fruits (Chou *et al.*, 1987; Kim and Kim, 1987; Ohitani *et al.*, 1991; Pabst *et al.*, 1992) of *R.* spp. have been reported. Moreover, many flavonoids from their fruits have so far been reported (Henning, 1981; Do *et al.*, 1988). However, the study about roots of *R. crataegifolius* has not been reported.

Programmed cell death, apoptosis, appears to be an essential physiological process which plays an important role during the development of most multicellular organism, organ involution, and in many diseases, including cancer (Steller *et al.*, 1995). Apoptosis is characterized by nuclear condensation, fragmentation, and degradation of DNA into oligonucleosome fragments, (Steller *et al.*, 1995; Im *et al.*, 1999). In cancer treatment, it is clear that many anticancer drugs induce apoptosis in cancer cells.

Topoisomerase is a family of enzymes that are essential for a variety of DNA-associated process such as replication, transcription, and recombination (Liu, 1989; Chen and Liu, 1994). The two primary eukaryotic DNA topoisomerase I and II have functions in similar but distinct fashions to modify DNA conformation. Topoisomerase I catalyzes DNA relaxation via a transient single-strand break, with covalent linking of the enzyme to the 3'-phosphotyrosyl end of the broken DNA strand (Pramod *et al.*, 1998).

This study is aimed to examine the activity of crude extract of *R. crataegifolius* roots in MCF-7 human breast carcinoma cells as a potent apoptosis inducer and DNA topoisomerase I inhibitor.

MATERIALS AND METHODS

Plant material

Roots of *R. crataegifolius* were collected at the Mt. Kumjung, Pusan, Korea in July, 1998. The plant was identified by

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the taxonomist, Prof. Jong Hee Park (Dept. of Pharmacy, Pusan National University, Korea) and the voucher specimen was deposited in the author's laboratory. The plant was washed with fresh water and air dried in the shade.

Extraction and fractionation

The dried material (2.3 kg) was extracted with methanol at room temperature. The methanol extract (MeOH ext, 100 g) was partitioned between water and butanol. The butanol soluble fraction was further partitioned into butanol (BuOH fr, 850 mg) and ethylacetate (EtOAc fr, 3.1 g) fractions, respectively. The aqueous layer of the butanol soluble fraction was further extracted with methanol to afford methanol soluble (W-M fr, 3.2 g) and methanol precipitated layer (W-ppt fr, 2.3 g).

Cell culture and growth study

MCF-7 human breast carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD, USA). The MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 50 mg/ml gentamicin, and 135 mg/ml glutamine at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed three times per week. The number of cells was counted with a hemacytometer. Cell growth was measured by the MTT colorimetric dye reduction method as described previously (Im *et al.*, 1999).

Assay of growth inhibition

Cells were seeded at an initial density of 1,000 cells per cm^2, incubated for 24 h, and treated with 100, 200, and 300 μg/ml W-M fr for 48 h, respectively. At the termination of the culture, cells were detached with 0.01% trypsin solution in phosphate-buffered saline and cell number was determined by hemacytometer counts of tryphan blue-excluding cells.

Cell cycle analysis

Cell cycle analysis using flow cytometry was performed as described previously (Oh *et al.*, 1999). For flow cytometry studies, 1×10^6 cells were suspended in 0.8 ml of PBS, fixed with ice-cold 70% EtOH at 4°C for 30 min, and washed with 1% bovine serum albumin (BSA)-PBS. Cells were resuspended in 1 ml of PBS containing 11 Kunitz/ml RNase (Sigma, St. Louis, MO, USA), incubated at 4°C for 30 min, washed once with BSA-PBS, and resuspened in propidium iodide (PI) solution (50 μg/ml). After cells were incubated at 4°C for 30 min in the dark, washed with PBS, and filtered through a 20 μm Nytex mesh. DNA content was measured on a FACS flow cytometry system (Becton Dickinson, San Jose, CA, USA) and data were analyzed using the Modfit software.

Agarose gel analysis of DNA fragmentation

For the qualitative analysis, the cells were harvested, washed with cold PBS, and lysed with lysis buffer (5 mM Tris-Cl, pH 7.4, 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100) at 4°C for 30 min. After centrifugation at 27,000 g for 15 min, supernatant was collected and incubated with proteinase K (300 μg/ml) during overnight and then extracted with phenol-chloroform. The DNA was precipitated with 0.1 volume of 5 M NaCl and 1 volume of isopropanol. The dried DNA pellet was resuspended in distilled water and treated with 300 μg/ml RNase A (Sigma). Electrophoresis of the DNA was performed on a 1.5% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.01 M EDTA). The agarose gel stained with ethidium bromide and the DNA was visualized on a UV transilluminator. The size of DNA was estimated by comparing with a standard 1kb DNA ladder.

Topoisomerase I relaxation assay

The activity of topoisomerase I was measured by the relaxation of superhelical plasmid DNA as previously described (Kim *et al.*, 1999a; Kim *et al.*, 1999b). The reaction mixture (20 μl each) containing 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml BSA, 3.75 μg/mlpBR322 plasmid DNA, topoisomerase I and various amounts of samples were incubated at 30°C for 30 min. The reactions were stopped by the addition of 5 μl of mixture of 50% glycerol/5% SDS containing 1 mg of bromophenolblue (BPB) per ml.

Statistics

Statistical significance was determined using Student's *t* test. $P<0.05$ was judged to be statistically significant.

RESULTS

Growth inhibitory effects

MTT assay was performed to test the effects of the methanol extracts of *R. crataegifolius* and their fractions on the viability of MCF-7 cells. MCF-7 cells were treated with each sample at the concentration of 200, 400, 600, 800 and 1000 μg/ml for 48 h, respectively. The methanol extract and their fractions showed anti-proliferative effect on MCF-7 cells in a concentration-dependent manner, and W-M fraction of their fractions had the most strong growth inhibitory effect (Fig. 1). Moreover, the relative number of cells treated with W-M fraction also decreased significantly (Fig. 2). Microphotographs of MCF-7 cells treated with W-M fraction were shown in Fig. 3. There were morphological changes and cell viability was decreased. Dimethylsulfoxide (DMSO) used as vehicle had no effect on the growth of MCF-7 cells (data not shown).

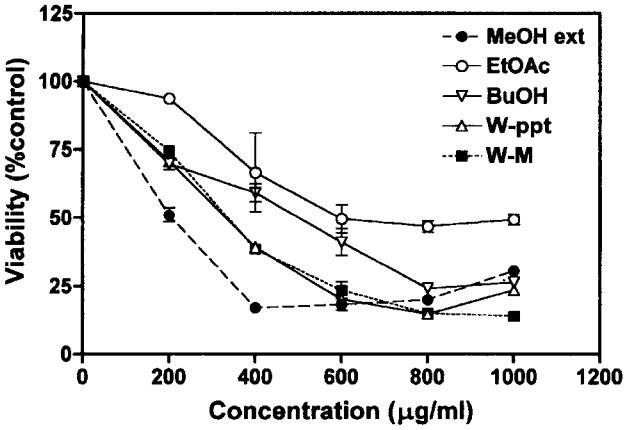

Fig. 1. Effects of methanol extract of *R. crataegifolius* and their fractions on the viability of MCF-7 cells after treatment for 48 h. Results are means ± S.D. from three separate experiments. MeOH ext, methanol extract; EtOAc, ethylacetate extract; BuOH, butanol extract; W-ppt, methanol precipitated layer; W-M, methanol soluble extract

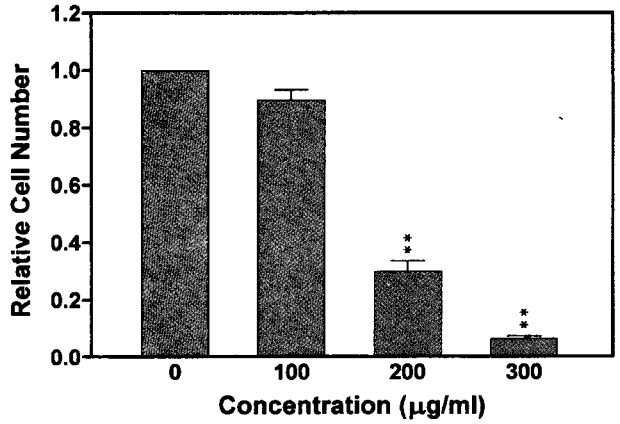

Fig. 2. Effect of W-M fraction on the growth of MCF-7 cells. Cells were seeded at an initial density of 1,000 cells per cm^2, incubated for 24 h, and treated with W-M fraction at 100, 200 and 300 μg/ml concentrations for 48 h. Cell number was determined by hemacytometer counts of tryphan blue-excluding cells. Each point represents mean ± S.D. of the triplicate determinations

Cell cycle analysis and DNA fragmentation

To determine whether the anti-proliferative effect of W-M fraction was caused by specifically perturbing cell cycle-related events, a set of experiments was performed to measure cell cycle distribution by flow cytometric analysis. The results showed that W-M fraction-treated MCF-7 cells led to a marked accumulation of cells in G_0/G_1 phase after treatment for 24 h and sub-G_1 apoptotic fraction was increased compared with untreated control after treatment for 48 h (Fig. 4 and Table I). Moreover, in cells treated with 200 and 300 μg/ml of W-M fraction for 48 h, DNA fragmentation was detected (Fig. 5), which is one of apoptotic characteristics. These results suggested that the growth inhibitory effect of W-M fraction was the result of arrest in G_1 phase and apoptosis.

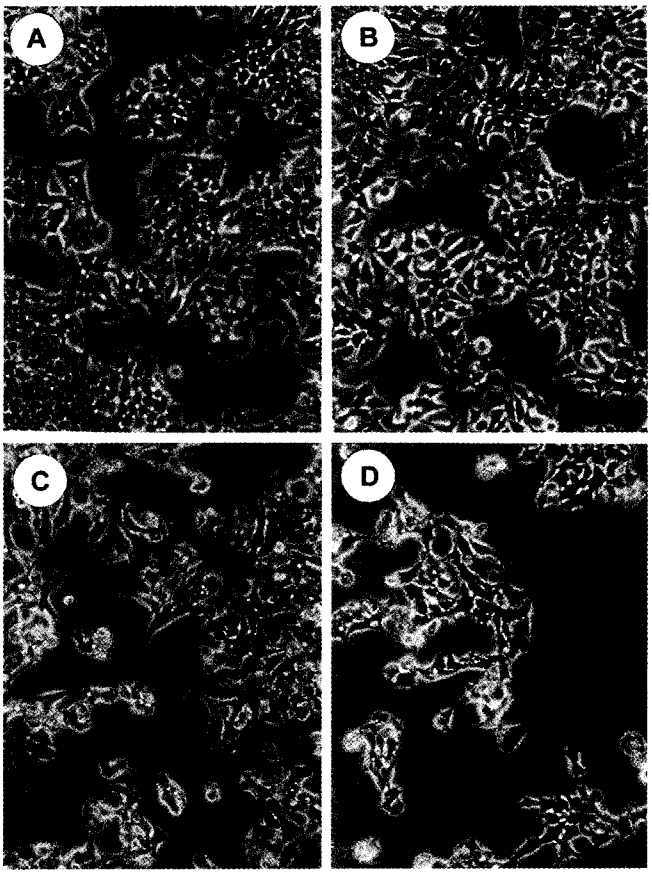

Fig. 3. Microphotographs of MCF-7 cells cultured with W-M fraction of *R. crategifolius*. MCF-7 cells were treated for 48 h. (A) control, (B) 100 μg/ml, (C) 200 μg/ml, and (D) 300 μg/ml. Phase contrast ×100

Topoisomerase I inhibition

The ability of the methanol extract and W-M fraction to inhibit DNA topoisomerase I was determined in the relaxation assay as demonstrated in Fig. 6. Topoisomerase I enzyme relaxed the supercoiled DNA, pBR322. However, the addition of methanol extract or W-M fraction inhibited DNA relaxation by topoisomerase I concentration-dependently. DMSO used as vehicle had no effect on DNA relaxation.

DISCUSSION

These studies showed that the methanol extract of roots of *R. crataegifolius*, used as a traditional medicine, inhibited the proliferation of MCF-7 human breast carcinoma cells. The methanol extract inhibited the growth of MCF-7 cells in a concentration dependent manner. More-

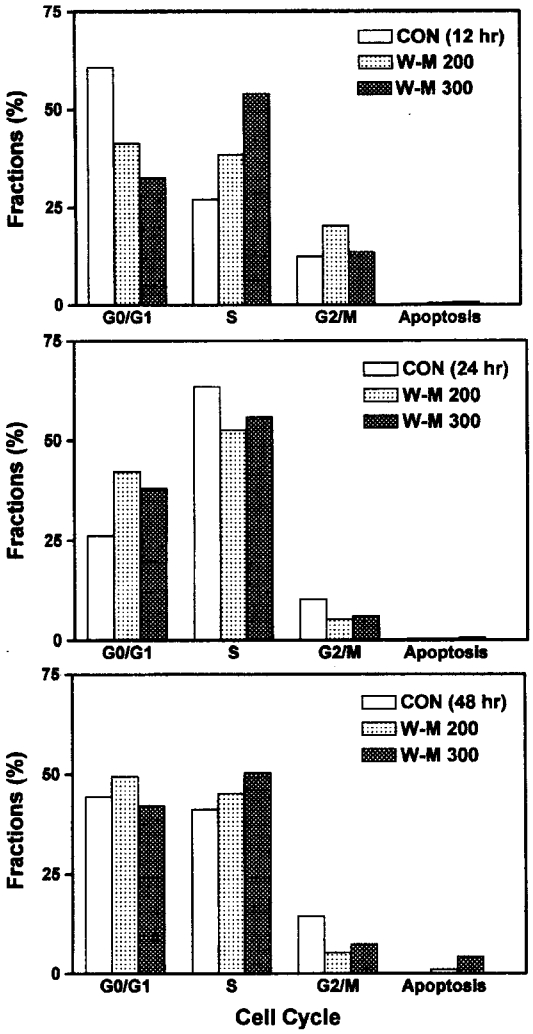

Fig. 4. Effect of W-M fraction in cell cycle. CON, control; W-M 200, treated with W-M fr 200 μg/ml; W-M 300 μg/ml, treated with W-M fr 300 μg/ml

Table I. Increase of sub-G_1 fraction of MCF-7 cells treated with W-M fraction of *R. crataegifolius* analysed by flow cytometry

Conditions	Sub-G_1 fraction	
	After 24 h	After 48 h
Untreated control	0.24	0.21
Treated with 200 μg/ml W-M fr	0.39	1.02
Treated with 300 μg/ml W-M fr	0.59	4.23

over, the W-M fraction, one of their solvent fractions, had the most cytotoxic effect on MCF-7 cells compared with other fractions. This inhibitory effect of W-M fraction on the viability of MCF-7 cells was achieved through inducing early G_0/G_1 phase arrest and apoptosis, by observing increase of apoptotic fraction in cell cycle analysis and internucleosomal DNA fragmentation. Both the methanol extract and W-M fraction had the inhibitory effect on the

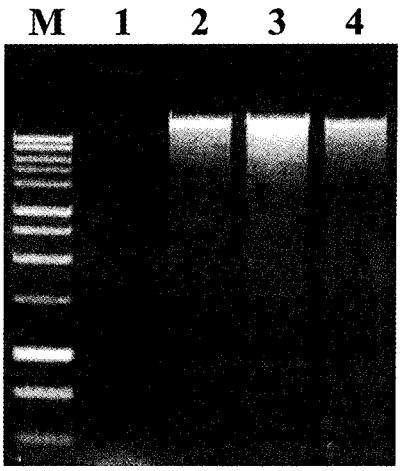

Fig. 5. Effect of W-M fraction on DNA fragmentation in. MCF-7 cells were treated with W-M fraction for 48 h. M, marker; 1, untreated control; 2, W-M fr 100 μg/ml; 3, 200 μg/ml; 4, 300 μg/ml

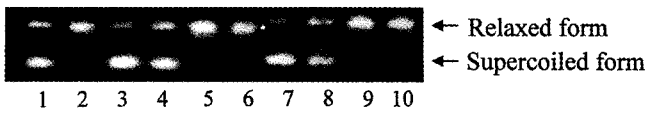

Fig. 6. Inhibition of topoisomerase I-mediated DNA relaxation by methanol extract and W-M fraction of *R. crataegifolius*. Topoisomerase activity was measured by the relaxation of superhelical plasmid DNA. The assay mixture (20 μl) contained pBR322 (20 μg/ml), topo I, and various amounts of the methanol extract and W-M fraction. After 30 min at 30, the reactions were stopped by the addition of 5 of stop solution. The samples were then loaded onto the agarose gel (0.8%) for electrophoresis followed by photography. Lane 1, pBR322; 2, topo I; 3, methanol ext 5 μg/ml; 4, 5 μg/ml; 5, 5 ng/ml; 6, 5 pg/ml; 7, W-M fr 5 μg/ml; 8, 5 μg/ml; 9, 5 ng/ml; 10, 5 pg/ml

DNA relaxation induced by topoisomerase I enzyme, concentration dependently. From this result, the inhibitory activity of them on topoisomerase I may partly participate in their cytotoxicity.

DNA topoisomerase I is an attractive molecular target for anticancer chemotherapeutic agents. According to previous reports, MCF-7 cells treated with camptothecin demonstrated very different cell cycle and apoptotic responses. Camptothecin is a known topoisomerase I poison (Hsiang *et al.*, 1985) that results in DNA breaks (Nelson and Kastan, 1994), cell cycle arrests at G_1, S, and G_2/M cell cycle checkpoint (Kastan *et al.*, 1991), and apoptosis (Solary *et al.*, 1994). Topoisomerase I inhibitors interfere with the enzymatic DNA-cleaving-reunion reaction by stablizing a DNA-topoisomerase I complex in which DNA is broken and the enzyme is co-valently linked to DNA strands. Other studies have indicated that cytotoxicity induced by camptothecin is correlated with S- and G_2-phase abnormalities in colon-carcinoma cell lines (Goldwasser *et al.*, 1995, 1996). Camptothecin derivative

CPT-11 (irinotecan), a topoisomerase I inhibitor, is a promising agent used to treat a variety of solid tumors including colorectal cancer and lung cancer (Masuda *et al.*, 1992; de Forni *et al.*, 1994; Cunningham, 1996; Oshita *et al.*, 1997; Cunningham *et al.*, 1998; Kudoh *et al.*, 1998). CPT-11 has been shown to induce apoptosis following triggering of DNA damage in the mechanism of cell killing in lung cancer cells (Ohmori *et al.*, 1993). A recently completed multicenter randomized clinical phase II trial reported that CPT-11 increased the 1-year overall survival rate 2.6 times greater than supportive care in metastatic colorectal cancer patients who had failed conventional treatment with 5-FU (Cunningham *et al.*, 1998). Sogawa *et al.* (2000) recently reported that a marine microalgal polysaccharide induced apoptosis and inhibited DNA topoisomerase I in human myeloid leukemia K-562 cells. Although the detailed molecular mechanism of apoptosis-inducing activity of *R. crataegifolius* roots extracts is beyond the scope of this investigation, we can assume the mechanism depending on the data of previous studies. The potent cytotoxic effects of W-M fraction and methanol extract of *R. crataegifolius* roots may result from their inhibitory effects on topoisomerase I because the topoisomerase I inhibition is known to trigger apoptotic cell death (Sogawa *et al.*, 2000; Cusack *et al.*, 2000).

Finally, the mechanisms of action of W-M fraction and methanol extract on cell cycle arrest and apoptosis in MCF-7 cells have not been known yet, but these fraction are valuable materials to be studied further on the cytotoxic effects on breast carcinoma cells.

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