Inhibitory Effect of Hot-Water Extract from Dried Fruit of *Crataegus pinnatifida* on Low-Density Lipoprotein (LDL) Oxidation in Cell and Cell-Free Systems

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The dried fruit of *Crataegus pinnatifida*, a local soft drink material and medical herb, was found to possess potential against oxidative stress. In the preliminary study, the antioxidant potential of a hot-water extract obtained from the dried fruit of *C. pinnatifida* (CF-H) was evaluated in terms of its capacity of quenching 1,1-diphenyl-2-picrylhydrazyl free radicals (EC_{50} = 0.118 mg/mL). After content analysis, it was found that CF-H is mainly composed of polyphenols including flavonoids (6.9%), procyanidins (2.2%), (+)-catechin (0.5%), and (−)-epicatechin (0.2%). The antioxidative bioactivity of CF-H had been assess previously using the models of $CuSO_4$ as cell-free system and sodium nitroprusside (SNP) plus macrophage RAW 264.7 cells as cell system to induce human low-density lipoprotein oxidation. CF-H was found to inhibit relative electrophoretic mobility and thiobarbituric acid reactive substances at the concentration of 0.5−1.0 mg/mL in the cell-free system and at 0.01−0.10 mg/mL in the cell system. Furthermore, it was found that CF-H decreased the SNP-induced cell lipid peroxidation and reduced glutathione depletion.

KEYWORDS: *Crataegus pinnatifida*; antioxidation; low-density lipoprotein; RAW 264.7 cells

INTRODUCTION

Oxidative stress can originate from several internal and external sources, such as metabolic reactions, virus infections, and dietary intake or cigarette smoking. Excessive formation of oxidants in biological systems and the consequent oxidative damage are topics of growing interest in recent years, because these processes probably contribute to several diseases such as atherosclerosis and cancer. It is reported that oxidative LDL, which could be induced by free radicals, enhanced the uptake of lipoprotein by macrophages, leading to cellular cholesterol accumulation and foam cell formation, the hallmark of early atherosclerosis lesions (*1*). Many studies have supported the hypothesis that antioxidant nutrients and/or medicines play a protective role in human health (*2−5*). The fruits of *Crataegus pinnatifida* (Rosaceae) have been used traditionally as a peptic agent in oriental medicine and recently as a local soft drink material for the diet. In addition, it is believed that preparation of leaves or fruits of *C. pinnatifida* improve heart function, and there are indications for declining cardiac performance, defi-

ciency in coronary blood supply, and mild forms of arrhythmia (*6*, *7*). However, other bioactivities remain unclear.

Atherosclerotic vascular disease is the leading cause of death among people with a Western lifestyle. The oxidative modifications of low-density lipoprotein (LDL) have been accepted as an important initial event in the development of atherosclerosis (*8*). LDL may be oxidized in vitro by several cell types (endothelial cell or macrophage) or by a cell-free system (metal ions or azo initiators) (*9*). Activated macrophages produced massive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that might accelerate LDL oxidation. Lipoprotein oxidation is unlikely to occur in plasma having high concentrations of antioxidants and proteins that may chelate metal ions. Phenolic compounds are antioxidant bioactive substances occurring widely in food plants (*10*, *11*) and, therefore, potentially present in the human plasma in a diet-dependent concentration. This study showed that a hot-water extract obtained from the dried fruit of *C. pinnatifida* (CF-H) containing phenolic compounds might possess antioxidant potential. To investigate the antioxidant properties of the dried fruits of *C. pinnatifida*, we established the model of cell-mediated LDL oxidation by adding sodium nitroprusside (SNP) to RAW 264.7 cells and used copper sulfate to induce LDL

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oxidation as a cell-free system. The results demonstrated that CF-H inhibited oxidation of LDL in both cell and cell-free systems.

MATERIALS AND METHODS

Chemicals. Ethylenediaminetetraacetic acid (EDTA), sodium chloride, SNP, cupric sulfate, sodium dodecyl sulfate (SDS), polyacrylamide, Coomassie blue, thiobarbituric acid, trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane, catechin, and (−)-epicatechin were purchased from Sigma (St. Louis, MO). A PD-10 column was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). The Paragon lipoprotein (Lipo) electrophoresis kit was purchased from Beckman Coulter (Brea, CA).

CF-H Preparation and Components Quantitation. Dried fruit of *C. pinnatifida* weighing 360 g were extracted twice with 1000 mL of water at 100 °C for 1 h. The extracted solution was filtered and then condensed to obtain ∼88 g of CF-H. We determined total flavonoids content according to the Kawaii method (*12*) using rutin as a standard. Total procyanidins content was analyzed according to the description of Kartring et al. (*13*).

HPLC Analysis. HPLC analyses were performed using a Hitachi L7100 system with a 5 μm ODS-Hypersil column (250 × 4.6 mm). The mobile phase was 24% of methanol and 76% of 0.5% (v/v) orthophosphoric acid in water. The detection wavelength was set at 280 nm, and the flow rate was set to 1.0 mL/min. Quantitation was carried out by the external standard method on the basis of area at 280 nm using calibration curves of (+)-catechin and (−)-epicatechin.

Determination of Free Radical Quenching Capacity. The free radical quenching capacities of CF-H, (+)-catechin, and (−)-epicatechin were tested according to a method of bleaching stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) (*14*). A reaction mixture containing methanol (3 mL), DPPH (10 mM, 30 μL), and various concentrations of tested sample was allowed to stand at room temperature for 30 min before mixing with redistilled water (1 mL) and toluene (3 mL). The solution was then centrifuged, and the absorbance of the upper phase was read at 517 nm against a blank without CF-H and processed alike.

Isolation of LDL. Blood was obtained from healthy volunteers in the presence of 0.01% EDTA. LDL (1.019−1.063 g/mL) was isolated by sequential density ultracentrifugation at 4 °C in an Optima TL Beckman ultracentrifuge (Beckman Instruments, Mountain View, CA) as described previously (*15*). After the isolation, EDTA existing in LDL was removed by a Sephadex G-25 column (Pharmacia PD-10; bed volume = 9.1 mL) equilibrated with phosphate-buffered saline (PBS). The protein content was measured using the Coomassie blue reagent (Pharmacia).

Cell Culture. Rat macrophage RAW 264.7 cells were grown in a culture flask in RPMI 1640 containing fetal bovine serum (10%) in an incubator regulated with 5% carbon dioxide and 95% air. The cells were plated at a density of 5×10^5 cells/mL and incubated overnight before being replaced with DME-F12 medium. All oxidation experiments were performed in DME-F12. Cell viability was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) as described in Sigma product literature.

LDL Oxidation. LDL was diluted in 10 mM PBS and incubated at 37 °C in the presence of 10 μM CuSO₄ or 0.1 mM SNP in RAW 264.7 culture for oxidation. After the pretreatment with CF-H, oxidation of LDL was carried out and electrophoretic mobility and lipid peroxidation of the LDL were then measured as described below.

Electrophoretic Mobility. The electrophoretic mobility of native or oxidized LDL was detected by agarose gel electrophoresis (*16*). The experiment was performed using a Beckman Paragon Lipo gel electrophoresis system (Beckman Analytic, Milan, Italy). Briefly, the agarose gel (0.5% agarose and 1.0% barbital buffer) was electrophoresed (100 V) in a buffer containing 10 mmol/L 5,5-diethylbarbituric acid and 50 mmol/L 5,5-diethylbarbituric acid sodium salt for 30 min. After electrophoresis, the lipoproteins in the gel were immobilized in a fixative solution (containing 60% absolute alcohol, 30% deionized water, and 10% glacial acetic acid), and the gel was dried to a film. The lipoprotein pattern was visualized by staining the film with a lipid-specific stain. This result was expressed as the distance away from the origin.
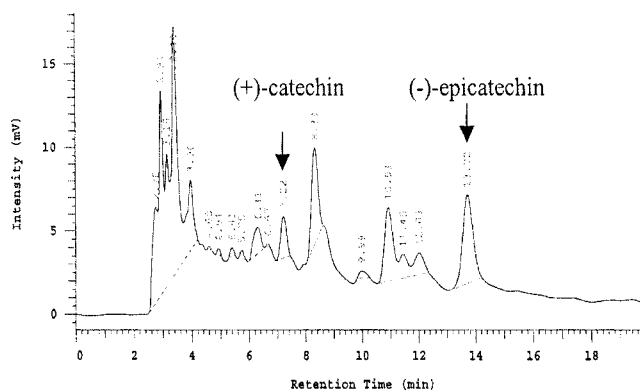
Figure 1. HPLC analysis of hot-water extracts of the dried fruit of *C. pinnatifida* (CF-H) recorded at 280 nm. For other conditions, see Materials and Methods.

Thiobarbituric Acid Reactive Substances (TBARS). TBARS assay was performed according to the procedures of Camejo et al. (*17*). To each tube containing 0.55 mL of the incubated LDL was added 0.5 mL of 25% (w/v) TCA to denature protein. After the samples had been centrifuged (10000 rpm) at 10 °C for 30 min to remove pellets, 0.5 mL of 1% thiobarbituric acid (TBA) in 0.3% NaOH was added to the supernatant, and the mixed reagents reacted at 90−95 °C for 40 min in the dark. After completion of the reactions, samples were detected with excitation at 532 nm and emission at 600 nm in a Hitachi F2000 spectrophotofluorometer. The concentration of TBARS is expressed as equivalents of 1,1,3,3-tetraethoxypropane that was used as standard.

Lipid Peroxidation and GSH Assay of Macrophage. RAW 264.7 cells were treated with SNP and/or CF-H. The lipid peroxidation product, malondialdehyde (MDA), was assayed according to a fluorometric method described by Yagi et al. (*18*). The results were expressed as MDA formation per milligram of cell protein.
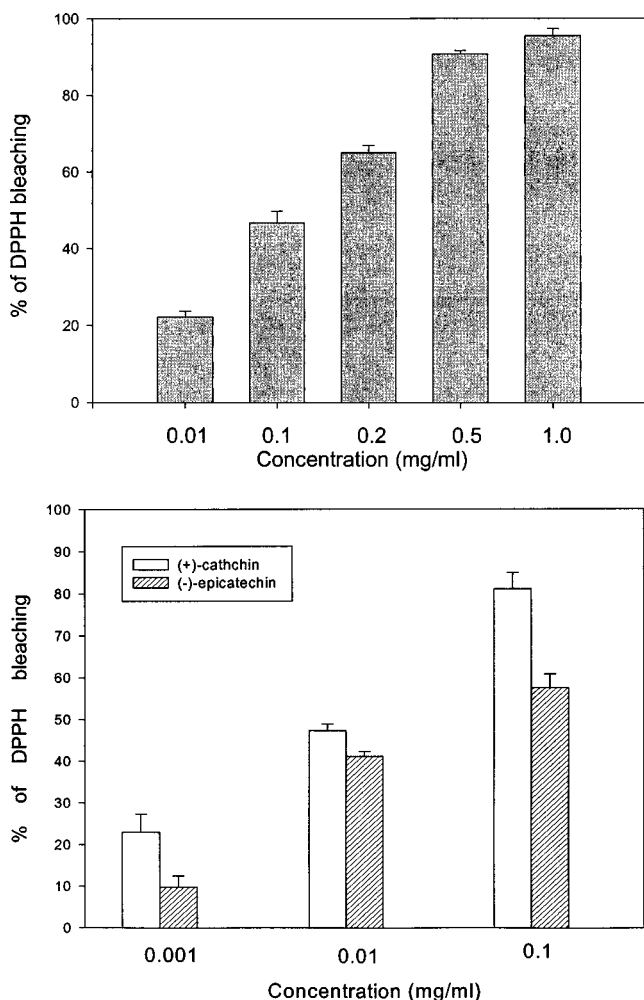
The reduced form of glutathione (GSH) content was assayed according to the method of Hissin and Hilf (*19*). In brief, the cell was homogenized with phosphate buffer containing 25% metaphosphoric acid (pH 8). After centrifugation (100000*g*, 30 min), *o*-phthaladehyde was added to the supernatant and incubated for 15 min at room temperature. Fluorescence at 420 nm was determined with the excitation at 350 nm. The results were expressed as micrograms of GSH per milligram of cell protein.

Statistics and Presentation. Data are presented as mean ± SD of three separate experiments performed in triplicate. Differences between treatments were analyzed by ANOVA, and the minimum level of significance was defined at $P < 0.05$.

RESULTS

Concentrations of Extract Components of CF-H. To establish the composition of CF-H extracted from dried fruit of *C. pinnatifida*, the total concentrations of flavonoids and procyanidins were determined. CF-H consists of 6.9% of flavonoids and 2.2% of procyanidins. From HPLC analysis, the content of (+)-catechin was 0.5% and that of (−)-epicatechin was 0.2% (**Figure 1**). The yield and composition of CF-H are highly consistent in each extract. The extract was stored at 4 °C and used in the following studies.

Free Radical Quenching Capacity of CF-H, (+)-Catechin, and (−)-Epicatechin. To determine the free radical quenching capacity of CF-H, the bleaching of DPPH by CF-H was measured. The result summarized in **Figure 2A** shows that CF-H was able to quench the DPPH free radicals in a dose-dependent manner. CF-H at the concentration of 0.1 mg/mL quenched ∼50% of free radicals. In addition, it showed that the free radical quenching capacity of (+)-catechin was more potent than that of (−)-epicatechin (**Figure 2B**).

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Figure 2. Effect of CF-H (**A**) and (+)-catechin and (−)-epicatechin (**B**) on quenching DPPH. Various concentrations of CF-H, (+)-catechin, and (−)-epicatechin were added, and results were analyzed by measuring the absorbance at 517 nm. Mean ± SD ($n = 3$).

Table 1. Effect of CF-H on the $CuSO_4$-Induced Oxidation of LDL

| CF-H (mg/mL)	Ox-LDL	REM[a]	TBARS (nmol/mg of LDL)
		1.00 ± 0.02	0.19 ± 0.02
	$CuSO_4$	5.28 ± 0.46	18.02 ± 0.46
0.10	$CuSO_4$	5.30 ± 0.04	18.15 ± 0.06
0.50	$CuSO_4$	$3.05 \pm 0.02^*$	$2.04 \pm 0.12^{**}$
1.00	$CuSO_4$	$1.25 \pm 0.02^*$	$1.22 \pm 0.02^{**}$

[a] LDL was pretreated with various concentrations of CF-H for 1 h and then incubated with $CuSO_4$ at 37 °C for 24 h. LDL oxidation was assessed by REM and TBARS as described under Materials and Methods. All data are presented as mean ± SD from three experiments. *, $P < 0.01$; **, $P < 0.001$, as compared with $CuSO_4$-treated alone.

Effect of CF-H on $CuSO_4$-Induced LDL Oxidation. The oxidation of LDL was executed by incubating LDL with 10 μM $CuSO_4$ for 24 h. Success in the induction of oxidized LDL was confirmed by the increased electrophoretic mobility as shown in **Table 1**. When the oxidation was carried out in the presence of CF-H (0.5 and 1.0 mg/mL), the relative electrophoretic mobility (REM) of LDL slowed significantly. The extent of lipid peroxidation in oxidized LDL mediated by copper incubation was assessed by TBARs assay. $CuSO_4$ increased the peroxidation of LDL lipid to 18-fold of the control. Treatment

Table 2. Effect of Sodium Nitroprusside on LDL Oxidation in Cell and Cell-Free Systems

treatment[a]			
RAW	SNP	REM	TBARS (nmol/mg of LDL)
−	−	1.00 ± 0.02	0.19 ± 0.02
−	+	$2.02 \pm 0.05^*$	$8.72 \pm 0.15^{**}$
+	−	1.20 ± 0.04	4.86 ± 0.82
+	+	$2.96 \pm 0.12^*$	$38.22 \pm 1.54^{***}$

[a] LDL was incubated with or without RAW 264.7 macrophage in the presence or absence of SNP at 37 °C for 20 h. REM and TBARS of LDL were determined as described under Materials and Methods. All data are presented as mean ± SD from three experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, as compared with the cell-free group and without SNP.
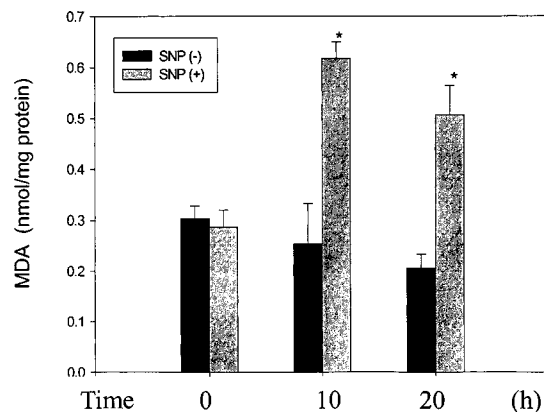

Figure 3. Effect of SNP-induced lipid peroxidation on the macrophage. The RAW 264.7 macrophage was incubated in the presence or absence of SNP (0.1 mM) at 37 °C for 10 and 20 h in DME-F12 medium, and then the cell was harvested and the formation of lipid peroxidation (MDA) determined. *, $P < 0.05$, compared with SNP-free group ($n = 3$).

of 0.5 and 1.0 (mg/mL) CF-H along with $CuSO_4$ reduced TBARs significantly (**Table 1**).

Effect of SNP on Macrophage-Mediated LDL Oxidation. SNP is a complex of ferrous iron with cyanide and nitric oxide. Although the mechanisms of SNP-oxidized LDL are complicated, it is suggested that SNP enhances macrophage-dependent LDL oxidation by an iron-dependent process (*20*). Superoxides released from the macrophage play an essential role in liberating iron from SNP. The iron can then oxidize LDL by a mechanism that probably involves endogenous hydroperoxide of LDL and/ or hydroperoxide generated by the cell. In addition, it is reported that nitric oxide released from SNP could combine with superoxide generated by the cells to produce peroxynitrite, which then initiates LDL oxidation (*21*). **Table 2** showed that SNP increased LDL oxidation in cell and cell-free systems as seen in the changes in REM and TBARS, but the increase in the cell system was more dramatic. In the presence of macrophage, LDL oxidation showed a smaller increase in both TBARS and REM in the absence of SNP. Therefore, we used SNP plus macrophage as the induction model of LDL oxidation. As seen in **Figure 3**, SNP increased significantly the formation of lipid peroxide in RAW 264.7 cells.

Effect of CF-H on SNP-Mediated Lipid Peroxidation and GSH Depletion in Macrophage. The MTT assay showed that the concentration of 0.5−2.0 mg/mL of CF-H exhibited a proliferation effect on RAW 264.7 cells (**Figure 4**). Therefore, 0.01−0.10 mg/mL of CF-H was used for the following cell system assay. It is reported that macrophage GSH content is inversely related to cell-mediated oxidation of LDL and that
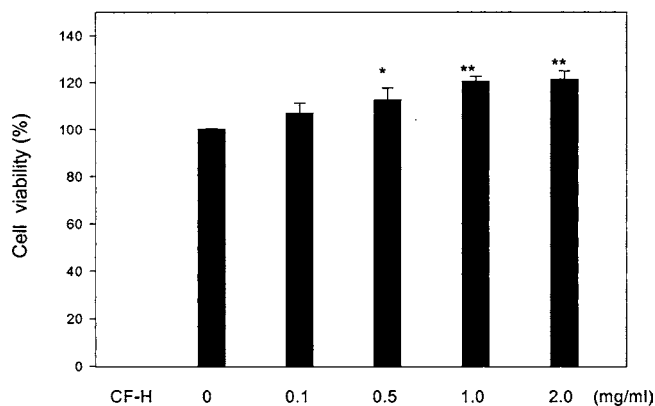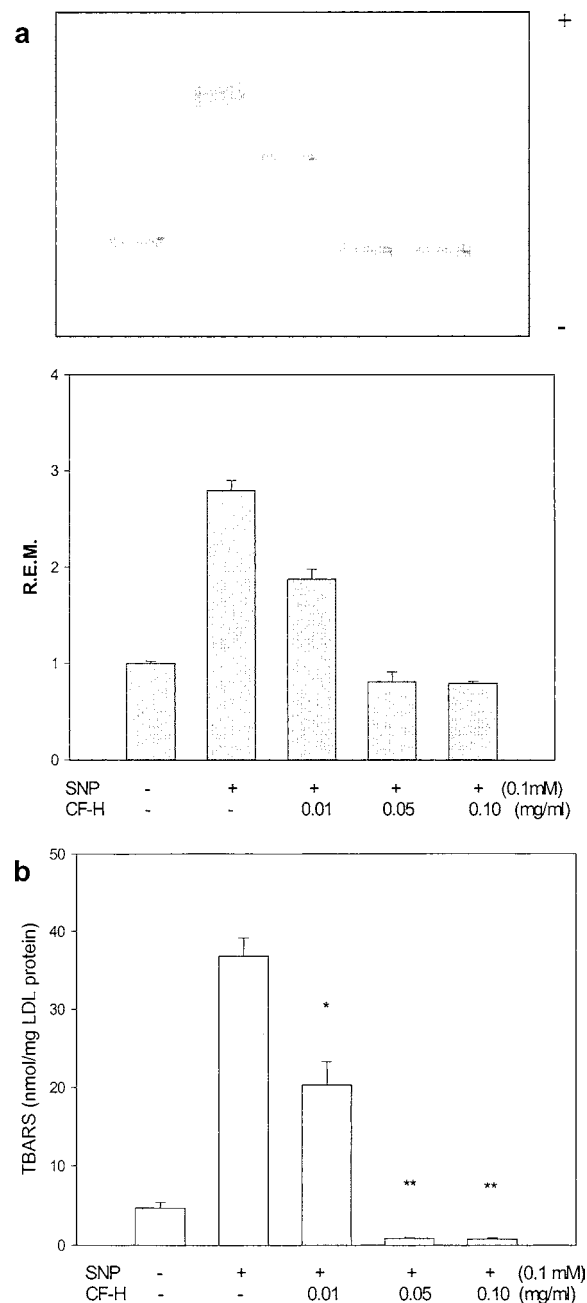
Figure 4. Cell viability assay of CF-H on the macrophage. The RAW 264.7 cell was treated with various concentrations of CF-H. The medium was changed after 20 h and incubated with MTT for 4 h. The medium was removed, and 2-propanol was added to dissolve the formazan crystal. The absorbance was determined at 563 nm. *, $P < 0.05$; **, $P < 0.01$, as compared with solvent-treated group ($n = 3$).
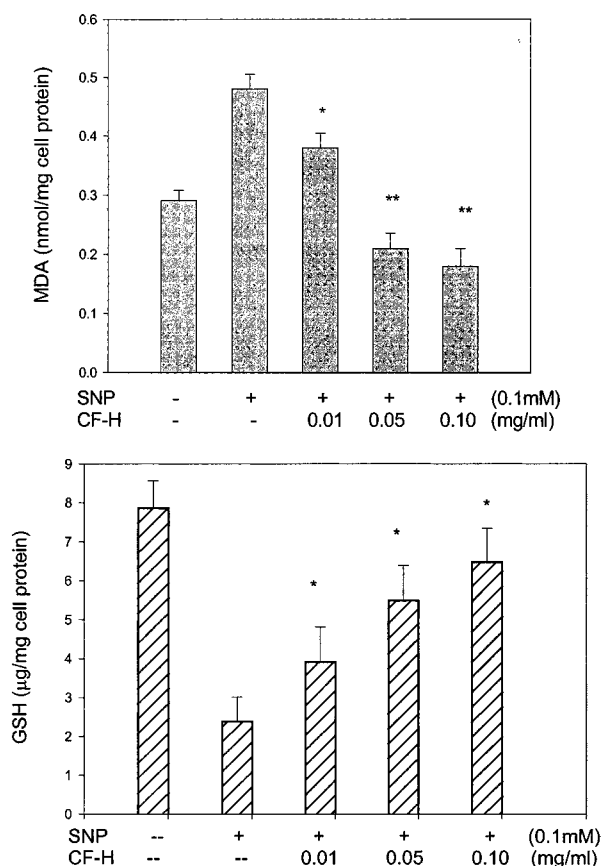
Figure 5. Effect of CF-H on lipid peroxidation and GSH content of RAW 264.7 cells treated with SNP. RAW 264.7 cells were incubated with CF-H and SNP at 37 °C for 20 h in DME-F12 medium. (**A**) The macrophage was assayed for lipid peroxidation formation (MDA). *, $P < 0.05$; **, $P < 0.01$, as compared with SNP-treated group ($n = 3$). (**B**) The cell extract was assayed for GSH as described under Materials and Methods. *, $P < 0.05$, as compared with SNP-treated group ($n = 3$).

Figure 6. Effect of CF-H on SNP-mediated macrophage-induced LDL oxidation. LDL was incubated with CF-H-pretreated (0, 0.01, 0.05, and 0.10 mg/mL) macrophages in the presence SNP (0.1 mM) at 37 °C for 20 h in DME-F12 medium, and REM and TBARS were then evaluated. (**A**) Agarose gel electrophoresis of LDL: (lane 1) native LDL; (lane 2) LDL incubated with SNP; (lane 3) SNP and 0.01, 0.05, or 0.10 mg/mL CF-H. (**B**) Lipid peroxidation of LDL: *, $P < 0.01$; **, $P < 0.001$, as compared with SNP-treated group ($n = 3$).
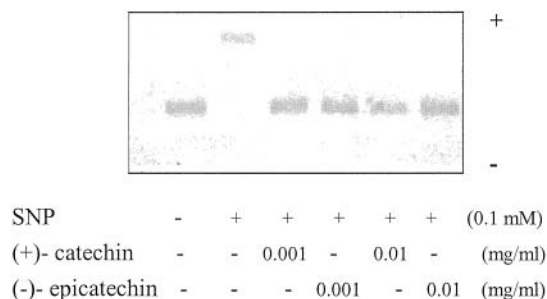
lipid peroxide enhances the cell-mediated oxidation of LDL. As seen in **Figure 5**, CF-H decreased significantly the SNP-induced lipid peroxidation and GSH depletion.

Effect of CF-H on SNP-Mediated Macrophage-Induced LDL Oxidation. As shown in **Figure 6**, CF-H demonstrated inhibitory effect against SNP-mediated macrophage-induced

LDL oxidation as evident in the variations in REM and TBARS. **Figure 6B** shows that 0.01 mg/mL of CF-H decreased the SNP-mediated macrophage-induced LDL oxidation significantly. In addition, TBARS of macrophage-mediated LDL oxidation free of SNP was 4.86 ± 0.82 nmol/mg of LDL protein; however, 0.05 mg/mL of CF-H treated showed 0.84 ± 0.09 nmol/mg of LDL protein. This means that 0.05 and 0.10 mg/mL of CF-H blocked completely the SNP-mediated macrophage-induced LDL oxidation. In addition, as shown in **Figure 7**, (+)-catechin and (−)-epicatechin at concentrations of 0.001 mg/mL and 0.01

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Figure 7. Effect of (+)-catechin and (−)-epicatechin on SNP-mediated macrophage-induced LDL oxidation. LDL was incubated with (+)-catechin- or (−)-epicatechin-pretreated (0, 0.001, and 0.01 mg/mL) macrophages in the presence of SNP (0.1 mM) at 37 °C for 20 h in DME-F12 medium, and REM was then evaluated.

mg/mL completely blocked SNP-mediated macrophage-induced LDL oxidation as evident in REM.

DISCUSSION

The present study showed that CF-H exhibited free radical scavenging property by DPPH assay and contained phenolic components. In addition, it has demonstrated the in vitro antioxidant activity of CF-H in inhibiting the oxidation of LDL by copper- and SNP-mediated macrophage systems. Several pieces of direct and indirect evidence have validated the use of copper as a prooxidant model compound. LDL isolated from atherosclerotic plaques share similar chemical and physical properties with copper-oxidized LDL (*22*). Antibodies raised against copper-oxidized LDL recognize antigenic epitopes located in atherosclerotic lesions (*23*). In epidemiological studies, high serum concentrations of copper are associated with an increased LDL oxidation in vivo and with an accelerated progression of the atherosclerotic disease (*24*). Finally, it has been reported that copper bound to ceruloplasmin may directly oxidize LDL or it can be released by prooxidant reactions occurring within the atherosclerotic plaque (*25*). Macrophage-mediated oxidation of LDL is thought to play a key role during early atherogenesis, and cellular oxygenases were shown to mediate this process. It is proposed that nitric oxide acting as a chain-breaking antioxidant can inhibit the progression of lipid peroxidation in cell-dependent oxidation of LDL. However, SNP, a NO donor, enhanced cell-mediated oxidation of LDL by a mechanism dependent on superoxide production and transition metal ions (*20*). The mechanism of SNP-mediated macrophage-induced LDL oxidation is complicated, but it produced an environment with oxidative stress that enhanced LDL oxidation. From these two systems, it is suggested that the inhibitory effect of CF-H on LDL oxidation might involve chelating ion or decreasing oxidative stress.

Several lines of evidence implicate oxidatively damaged LDL as an atherogenic agent (*26−28*). (1) Oxidized LDL exerts a multitude of potentially atherogenic effects in vitro and in vivo. (2) Lipoprotein-like particles with oxidative damage have been isolated from atherosclerotic lesions. (3) Lipid oxidation products such as MDA have been immunohistochemically detected in human and animal atherosclerotic lesions. (4) A number of structurally unrelated antioxidants retard lesion formation in hypercholesterolemic animals. Thus, it is reasonable to hypothesize that agents that could slow or prevent the oxidative process may be beneficial in lowering the incidence of atherosclerosis and coronary heart disease. Nonvitamin phenolic compounds are bioactive substances ubiquitous in food plants and, therefore, potentially present in human plasma in a diet-dependent

concentration. A number of these phenols acting as antioxidants may participate in antioxidant cell defenses either by scavenging efficiently both ROS and RNS or by recycling ascorbate (*29*) and therefore may exert an α-tocopherol "sparing effect" (*30*).

To date, more than 50 flavonoids have been isolated from *Crataegus* (*31*, *32*). Our content analysis showed the major phenolic components contained in CF-H were flavonoids. Consistent with previous reports (*11*, *33*, *34*), our results demonstrated that CF-H possessed antioxidant properties both in cell and cell-free systems of LDL oxidation. In addition, according to our study, CF-H contained (+)-catechin, (−)-epicatechin (monomer), and procyanidins (dimers to hexamers) that exhibited strong radical scavenging activity (*35*) and significantly inhibited Cu-induced LDL oxidation (*36*). Consistent with our results, **Figures 2B** and **7** showed that (+)-catechin and (−)-epicatechin possessed potent capacities in scavenging free radicals and blocking SNP-mediated macrophage-induced LDL oxidation. We suggest that CF-H possesses antioxidant activity for containing phenolic components. CF-H may inhibit LDL oxidation by an ion-chelating effect, scavenging free radicals and blocking the formation of cell lipid peroxides that can enhance LDL oxidation (*37*). In conclusion, CF-H exhibited an inhibitory effect on LDL oxidation in both cell and cell-free systems.

ABBREVIATIONS USED

CF-H, hot-water extract of the dried fruit of *C. pinnatifida*; DPPH, 1,1-diphenyl-2-picrylhydrazyl; LDL, low-density lipoprotein; REM, relative electrophoretic mobility; SNP, sodium nitroprusside; TBARS, thiobarbituric acid reactive substances.

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