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Antioxidant Property of an Ethanol Extract of the Stem of *Opuntia ficus-indica* var. *Saboten*

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An ethanol extract of the stem of *Opuntia ficus-indica* var. *saboten* (OFS) was assessed to determine the mechanism(s) of its antioxidant activity. The ethanol extract exhibited a concentration-dependent inhibition of linoleic acid oxidation in a thiocyanate assay system. In addition, the OFS extract showed dose-dependent free-radical scavenging activity, including DPPH radicals, superoxide anions ($O_2^{\bullet -}$), and hydroxyl radicals ($^{\bullet}OH$), using different assay systems. The OFS ethanol extract was also found to be effective in protecting plasmid DNA against the strand breakage induced by hydroxyl radicals in a Fenton's reaction mixture. Furthermore, the extract showed significant ($p < 0.01$) dose-dependent protection of mouse splenocytes against glucose oxidase-mediated cytotoxicity. Finally, the OFS extract was characterized as containing a high amount of phenolics (180.3 mg/g), which might be the active compounds responsible for the antioxidant properties of the OFS extract.

KEYWORDS: ***Opuntia ficus-indica* var. *saboten* (OFS); reactive oxygen species; antioxidant activity; phenolics**

INTRODUCTION

In recent years, there has been a global trend toward the use of natural phytochemicals present in natural resources, such as fruits, vegetables, oilseeds, and herbs, as antioxidants and functional foods (*1*−*3*). Natural antioxidants can be used in the food industry, and there is evidence that these substances may exert their antioxidant effects within the human body (*4*, *5*). The prickly pear cactus (*Opuntia ficus-indica*) has a global distribution and is an important nutrient and food source (*6*, *7*). About 1500 species of cactus are in the genus *Opuntia* and many of them produce edible and highly favored fruits. In addition, Mexicans have used *Opuntia* leaves and fruits for their medicinal benefits, such as for treating arteriosclerosis, diabetes, gastritis, and hyperglycemia (*8*−*10*). The prickly pear variety *Opuntia ficus-indica* var. *saboten* (OFS) is widely cultivated on Cheju Island, in southwestern Korea, and is used as a functional food in Korea (*11*, *12*). Previous study on crude compositions of the OFS showed that the major component of OFS is nitrogen free extract. In addition, OFS stem also contains more minerals and crude proteins and less crude fats than seed and fruit (*11*). More interestingly, the antioxidant activity of OFS is reported to correspond to well-known antioxidants, such as catalase, α-tocopherol, and ascorbic acid in a cell-free reactive oxygen species (ROS) generating system (*13*).

We previously demonstrated that the antioxidant activity of bioactive plant constituents might involve direct activity, such as scavenging free radicals, or indirect activity, which may be involved in chelating transition elements (*14*, *15*). Moreover, active constituents with strong reducing activity can also contribute to strong pro-oxidant activity (*16*, *17*). However, very little information is currently available on the active constituents present in OFS that are responsible for its antioxidant activity. Furthermore, the mechanism(s) of the antioxidant actions of OFS has yet to be determined. Hence, the purposes of this work were to determine the antioxidant property of OFS and to preliminarily define the active components and mechanism of its antioxidant action.

MATERIALS AND METHODS

Chemicals, Plastics, and Mice. Unless otherwise specified, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO), and all the plastics were from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). Inbred BALB/c mice (4−6 weeks old) were purchased from Damul Science (Yoosung, Korea).

Preparation of OFS Extract. OFS was obtained from a local market in Cheju Island. A 100-g sample of OFS stem was cut into small pieces and then freeze-dried to give a final weight of 18.7 g for crude OFS stem preparation. The crude preparation (10 g) was then mixed with 100 mL of absolute ethanol in a screw-capped flask and shaken at room temperature for 3 days to obtain ethanol extract. The resulting ethanol extract was subsequently filtered through filter paper (Whatman No. 3) and centrifuged at 5000*g* for 10 min. The collected supernatants were lyophilized to give a final weight of 1.23 g (2.3% of the initial amounts) for the dried powder of ethanol extract.

Antioxidant Activity of OFS Ethanol Extract against Lipid Peroxidation. The antioxidant activity of OFS against lipid peroxidation was measured through ammonium thiocyanate assay as described previously (*18*). The substrate solution containing 400 μL of ethanol extract (0.01 to 2 mg/mL distilled water), 200 μL of diluted linoleic acid (25 mg/mL 99% ethanol), and 400 μL of 50 mM phosphate buffer

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(pH 7.4) was incubated for 15 min at 40 °C. A 100-μL aliquot was then mixed with a reaction solution containing 3 mL of 70% ethanol, 100 μL of ammonium thiocyanate (300 mg/mL distilled water), and 100 μL of ferrous chloride (2.45 mg/mL 3.5% HCl). After the solution incubated at room temperature for 3 min, absorbance was measured at 500 nm.

DNA Nicking Assay. A DNA nicking assay was performed using supercoiled pBR322 plasmid DNA prepared from DH5α using Wizard Plus SV Minipreps (Promega, Madison, WI). Plasmid DNA (0.5 μg) was added to Fenton's reagents (30 mM H_2O_2, 50 μM ascorbic acid, and 80 μM $FeCl_3$) containing different concentrations of the ethanol extract, and the final volume of the mixture was brought up to 20 μL. The mixture was then incubated for 30 min at 37 °C and the DNA was analyzed on a 1% agarose gel followed by ethidium bromide staining.

Deoxyribose Assay. Deoxyribose assay to determine the rate constant for the reactions between either antioxidants and hydroxyl radicals (•OH) (referred as non-site-specific scavenging assay) or antioxidants and iron ions (referred as site-specific scavenging assay) was conducted as described by Halliwell et al. (*16*). For the non-site-specific scavenging assay, OFS extract of different concentrations was mixed with 1 mL of reaction buffer (100 μM $FeCl_3$, 104 μM EDTA, 1.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM L-ascorbic acid, pH 7.4) and incubated for 1 h at 37 °C. A 1-mL aliquot of 0.5% 2-thiobarbituric acid in 0.025 M NaOH and 1 mL of 2.8% trichloroacetic acid were added to the mixture and heated for 30 min at 80 °C. Finally, the mixture was cooled on ice and absorbance was measured at 532 nm using a spectrophotometer (Beckman, DU 530, Germany).

Site-specific scavenging activity, which represented the ability of OFS extract to chelate iron ions and interfere with hydroxyl radical generation, was measured using the same reaction buffer without EDTA. In addition, the ability of the extract to reduce the Fe^{3+}−EDTA complex to Fe^{2+}−EDTA complex, acting as pro-oxidant, was tested using the non-site-specific scavenging assay mixture without the addition of ascorbic acid.

Scavenging Activity onto DPPH Radicals. Scavenging activity on DPPH free radicals by the extract was assessed according to the method reported by Gyamfi et al. (*19*). Briefly, 50 μL of the ethanol extract containing varied amounts of powdered ethanol extract (1, 5, 10, and 50 μg/mL distilled water, respectively, in each reaction) was mixed with 1 mL of 0.1 mM DPPH−ethanol solution and 450 μL of 50 mM Tris−HCl buffer (pH 7.4). After the solution incubated for 30 min incubation at room temperature, reduction of DPPH free radicals was measured by reading the absorbance at 517 nm. In the experiment, L-ascorbic acid was used as positive control. The inhibition percent was calculated from the following equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

Scavenging Activity onto Superoxide Anions. Scavenging activity on superoxide radical ($O_2^{\bullet -}$) was assessed by the method described by Gotoh and Niki (*20*) with a slight modification. Briefly, different concentrations of the extract were added to the reaction solution containing 100 μL of 30 mM EDTA (pH 7.4), 10 μL of 30 mM hypoxanthine in 50 mM NaOH, and 200 μL of 1.42-mM nitro blue tetrazolium (NBT). After the solution was preincubated at room temperature for 3 min, 100 μL of 0.5 U/mL xanthine oxidase was added to the mixture and the volume was brought up to 3 mL with 50 mM phosphate buffer (pH 7.4). After the solution was incubated at room temperature for 20 min, absorbance was measured at 560 nm.

Cellular Assay for Antioxidant Activity. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were used for the evaluation of oxidative stress in cells on the basis of methods reported by Bass et al. (*21*) and Mosmann (*22*), respectively. In the experiments, mouse splenocytes and glucose/glucose oxidase (G/GO) were used as model cell and ROS generating systems, respectively. Briefly, stock solution of DCFH-DA (50 mM) (Calbiochem, Germany) was prepared in DMSO and stored in 100-μL aliquots in the dark at −20 °C. Splenocytes (10^7 cells/mL) were exposed to the G/GO system (10 mM D-glucose and 10 mU/mL glucose oxidase in RPMI 1640 medium) for varied time periods with or without the extract before incubating for 20 min with 25 μM DCFH-DA. Ten thousand events were counted per sample and the green fluorescence of DCF was recorded from 515 nm (FL I) using a FACS Vantage system (Becton-Dickinson, San Jose, CA). Cell viability was not affected by the incubation with 25 μM DCFH-DA. In addition, the cells were placed onto 96-well plates and exposed to the G/GO system for 4 h in the presence of the extract. At varied time points, 10 μL of MTT solution (5 mg/mL in PBS) was added to each well and the plates were incubated for an additional 4 h at 37 °C. Finally, 70 μL of acidic 2-propanol was added to each well, and the absorbance was measured at 560 nm using the SpectraCount ELISA reader (Packard Instrument Co., Downers Grove, IL).

Measuring DNA Synthesis and Cytotoxicity. Level of DNA synthesis by incubating splenocytes with OFS ethanol extract of different concentrations in the presence of 5 μg/mL of Concanavalin A (Con A) or lipopolysaccharide (LPS) was measured by adding 1 μCi of [*methyl*-^{3}H] Thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) to each well for the last 12 h during the 48-h culture period. Cells were then collected with a cell harvester (Inotech Inc., Switzerland), and the tritium contents were measured using a liquid scintillation counter (Packard). Cellular cytotoxicity induced by the treatment with OFS extract was measured using trypan blue exclusion assay (*23*). Briefly, splenocytes were cultured in RPMI 1640 supplemented with 0.5% FBS in the presence of 0.01 to 1 mg/mL OFS ethanol extract. At varied time points throughout the culture period (0−48 h), the cells were stained with 0.4% trypan blue and about 100 cells were counted for each treatment condition. Cytotoxicity was calculated as follows: % cytotoxicity = [(total cells − viable cells)/total cells] × 100.

Determination of Total Phenolics. Contents of total phenolics were determined according to the method of Folin−Ciocalteu reaction (*24*), using gallic acid as standard. Initially, the OFS extract (5 mg) was dissolved in 5 mL of methanol/water mixture (50:50, v/v), and the extract solution (500 μL) was mixed with 500 μL of 50% Folin−Ciocalteu reagent. The mixture was then allowed to stand for a 2−5 min period followed by the addition of 1.0 mL of 20% Na_2CO_3. After 10-min incubation at room temperature, the mixture was centrifuged for 5 min (1000*g*), and absorbance of the supernatant was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram sample.

Statistical Analyses. All data were expressed as 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

Scavenging Effects of OFS Extract on Fe^{3+}-Dependent Hydroxyl Radicals. To measure the scavenging activity of OFS extract on hydroxyl radicals generated in an Fe^{3+}-dependent manner, we initially measured the radical scavenging activity of OFS ethanol extract using an ammonium thiocyanate assay, which measures the degree of linoleic acid oxidation. As shown in **Figure 1**, OFS extract effectively inhibited Fe^{3+}-dependent linoleic acid oxidation in a dose-dependent manner such that 1.5−2 mg/mL of OFS extract inhibited oxidation by 50%. In contrast, a crude preparation of OFS stem inhibited oxidation only very weakly, even at the highest concentration tested (data not shown).

To further measure the scavenging effect of OFS ethanol extract on Fe^{3+}-dependent hydroxyl radicals, we investigated whether the extract reduced Fe^{3+}-dependent DNA nicking (**Figure 2**). When pBR322 plasmid DNA was dissolved in the reaction mixture, a time-dependent increase in the formation of single-stranded nicked DNA (Form II) and of double-stranded nicked and linear DNA (Form III) was observed (data not shown). However, the addition of 20 μg of OFS extract to the nicking reaction mixture increased Form I DNA formation. Consequently, the treatment caused Fe^{3+}-mediated Form III DNA formation to disappear and reduced Form II DNA

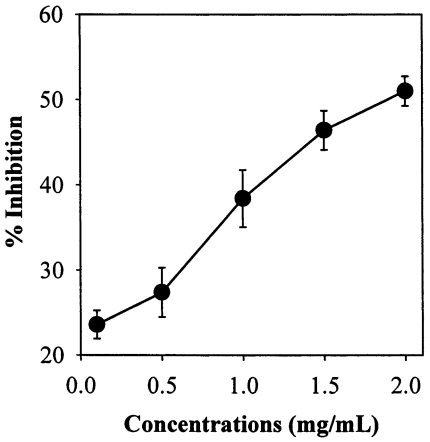

Figure 1. Inhibitory effects of OFS ethanol extract on hydroxyl radical-mediated linoleic acid oxidation. Hydroxyl radicals were generated by Fenton's reaction using an ammonium thiocyanate assay system, and the scavenging of hydroxyl radicals by OFS extract is expressed as the % inhibition. The concentration of OFS extract tested ranged from 0.1 to 2 mg/mL. The results are the means of three separate experiments.

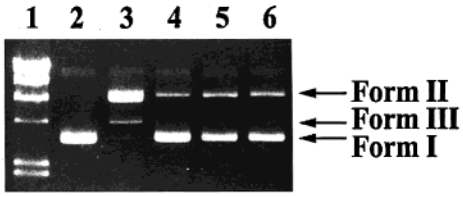

Figure 2. Inhibitory effects of OFS extract on DNA nicking caused by hydroxyl radicals. The DNA nicking reaction was initiated by adding 0.5 μg of pBR322 plasmid DNA to Fenton's reaction solution in the absence (lane 3) or presence (lane 4) of OFS extract for 30 min at 37 °C. Lanes 1 and 2 show the λ/*Hin*dIII DNA marker and native plasmid DNA, respectively. Lanes 5 and 6 show the results for reaction mixtures containing 2 U of SOD and 5 U of catalase, respectively.

formation (**Figure 2**, lane 4). This OFS ethanol extract-mediated antioxidant activity was similar to that of 2 U of superoxide dismutase (SOD) and 5 U of catalase, as shown in lanes 5 and 6, respectively. These results indicate that the OFS ethanol extract effectively mitigates the oxidative stresses on susceptible biomolecules, such as DNA.

To identify the mechanisms involved in OFS ethanol extract-mediated antioxidant activity, and particularly to determine whether the OFS extract decreases hydroxyl radical generation by chelating metal ions or by directly scavenging hydroxyl radicals, the effect of OFS extract on hydroxyl radicals generated by Fe^{3+} ions was measured by determining the degree of deoxyribose degradation, an indicator of thiobarbituric acid-malonaldehyde (TBA-MDA) adduct formation. As shown in **Figure 3**, concentration-dependent inhibition of hydroxyl radical-induced deoxyribose degradation was observed in both site-specific and non-site-specific assays. Relatively greater antioxidant activity was observed in the site-specific assay than in the non-site-specific assay when the same concentration of OFS extract was used, implying that OFS extract chelates metal ions rather than scavenging hydroxyl radicals directly.

In contrast, when the ability of OFS extract to reduce the Fe^{3+}−EDTA complex was tested to examine its pro-oxidant activity, there was weak pro-oxidant activity at low concentrations in the absence of ascorbic acid (**Table 1**), but not in the absence of both ascorbic acid and EDTA (data not shown). However, the pro-oxidant effect was not observed at high concentrations.

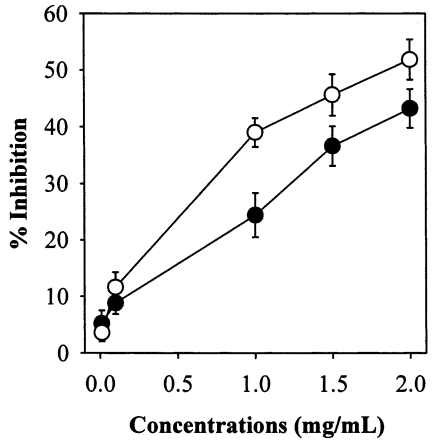

Figure 3. Inhibitory effects of OFS ethanol extract on hydroxyl radical-mediated deoxyribose degradation. Hydroxyl radicals were generated by Fenton's reaction using a deoxyribose assay system, and the non-site-specific (●) and site-specific (○) scavenging activities of hydroxyl radicals by OFS extract are expressed as the % inhibition. The concentration of RVS samples tested ranged from 0.01 to 2 mg/mL. The results are the means of three separate experiments.

Table 1. Pro-Oxidant Effect of the Ethanol Extract from OFS Stems on Iron-Dependent Hydroxyl Radical Generation[a]

amount of OFS ethanol extract (mg/mL)	optical density (A_{532} nm)	% stimulation[c]
control[b]	0.286 ± 0.005	
0.01	0.292 ± 0.007	2.1
0.1	0.319 ± 0.010	11.5
1	0.331 ± 0.011	15.7
1.5	0.282 ± 0.012	0
2	0.263 ± 0.006	0

[a] Experiments were conducted essentially as described by Halliwell et al. (*16*), except that ascorbic acid was omitted. [b] The control contained reaction buffer solution only. [c] The pro-oxidant effect of OFS extract was expressed as % stimulation, which was calculated as follows: % stimulation = $[(OD_{sample} - OD_{control})/OD_{control}] \times 100$. The results are the mean values of three separate experiments.

Scavenging Effect of OFS Extract on DPPH Radicals. Next, we tested the ability of OFS ethanol extract to scavenge DPPH radicals. As shown in **Figure 4**, the extract significantly inhibited the activity of DPPH radicals in a dose-dependent manner. Almost complete inhibition of the anti-DPPH radical activity was observed when 15 μg/mL of OFS extract was used, and the activity was similar to that of ascorbic acid, which was used as a control antioxidant. However, the concentration of OFS extract required to achieve a 50% reduction in DPPH radicals (IC_{50}), which was calculated using the concentration-activity curve, was 9.3 μg/mL, higher than that of ascorbic acid (3.2 μg/mL).

Scavenging Effects of OFS Extract on Superoxide Anions. We then tested the scavenging effect of OFS extract on superoxide anions by monitoring the reduction of NBT induced by superoxide anions produced by the xanthine oxidase-mediated degradation of hypoxanthine. **Figure 5** shows that OFS ethanol extract inhibited NBT reduction very efficiently. For example, OFS extract inhibited the production of superoxide anions by 75.7% when 1.5 mg/mL of the extract was added to the reaction solution. OFS extract alone did not change the absorbance of the reaction solution containing only NBT, suggesting that OFS extract did not directly reduce the NBT (data not shown).

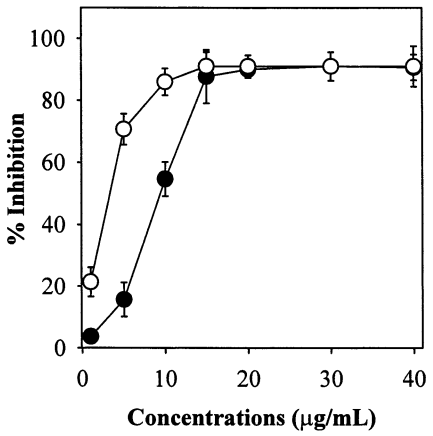

Figure 4. Free-radical scavenging activity of OFS ethanol extract measured using the DPPH assay. The direct scavenging activity of OFS extract (●) and ascorbic acid (○) on DPPH radicals is expressed as the % inhibition. The concentrations tested ranged from 1 to 40 μg/mL. The results are the means of three separate experiments.

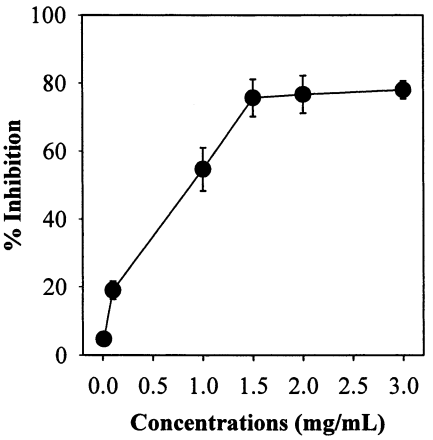

Figure 5. Inhibitory effect of OFS ethanol extract on NBT reduction. The inhibitory effect of OFS extract was tested by monitoring NBT reduction caused by superoxide anions using the hypoxanthine–xanthine oxidase system, as described in the Materials and Methods section. The concentration of OFS extract ranged from 0.01 to 3 mg/mL. The results are expressed as the mean values of triplicate experiments.

Protective Effects of OFS Extract on Splenocyte Injury induced by Glucose Oxidase-Mediated Radicals. We further investigated the antioxidant activity of OFS extract by measuring the degree of DCF formation (**Figure 6**) and the degree of cell injury (**Figure 7**) induced by hydroxyl radicals generated by the Haber-Weiss reaction in the G/GO system. As shown in **Figure 6**, the fluorescence intensity peak representing the DCF content clearly shifted to the right in the presence of G/GO, indicating the presence of intracellular ROS. In contrast, the shift was greatly reduced in the splenocytes incubated in the presence of 100 μg/mL of OFS extract. Similarly, OFS extract treatment significantly protected the splenocytes from G/GO-mediated cytotoxicity (**Figure 7**). For example, the addition of 50 and 100 μg/mL of OFS ethanol extract increased the viability of splenocytes by 69.9 ± 2.88 ($p < 0.05$) and 78.0 ± 3.05 ($p < 0.01$), respectively, compared to that of G/GO treatment alone (51.7%). These results are in accord with the previous observations that OFS extract contains active scavengers of both hydroxyl radicals and superoxide anions.

Effects of OFS Extract on DNA Synthesis in Splenocytes. Next, we determined the effect of OFS extract on DNA synthesis in splenocytes using a tritium incorporation assay. As shown

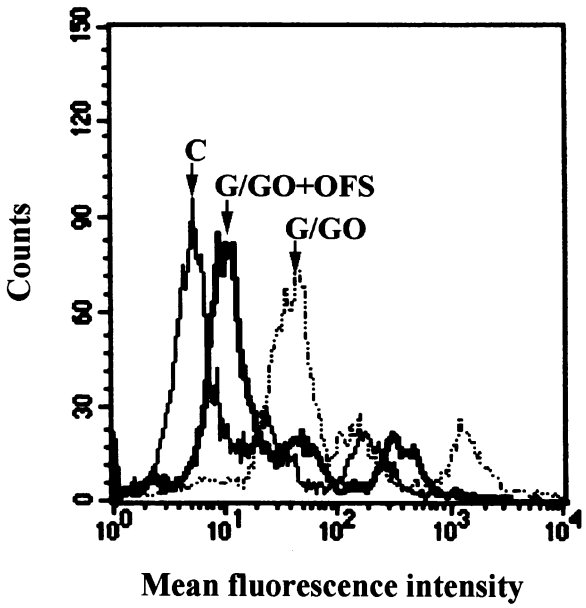

Figure 6. Flow cytometry analysis of splenocytes. Splenocytes were incubated using the G/GO system (10 mM glucose and 10 mU/mL glucose oxidase) in the presence of 100 μg/mL OFS extract for 4 h at 37 °C. DCFH-DA (25 μM) was then added for another 20 min, and fluorescence intensity was analyzed as described in the Materials and Methods section.

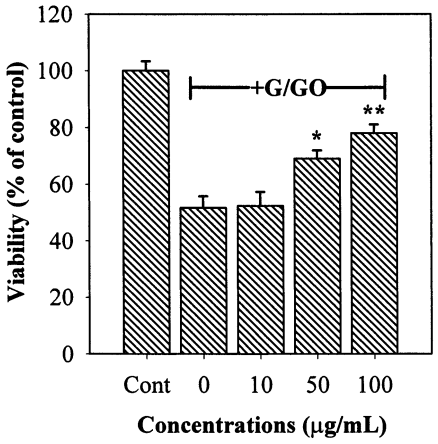

Figure 7. Protective effects of OFS extract on hydroxyl radical-mediated splenocyte death. Splenocytes were exposed to hydroxyl radicals (from the G/GO system, generated in the Haber–Weiss reaction) for 4 h in the presence of OFS extract. Each bar represents the mean ± SE of triplicate experiments and the figure shows a representative result from three separate experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences between the experimental and control values.

in **Figure 8**, the OFS ethanol extract showed dose-dependent inhibition of DNA synthesis in splenocytes stimulated with 5 μg/mL Con A, a T-cell mitogen. However, the OFS extract did not inhibit the proliferation of splenocytes stimulated with the same dose of LPS, a B-cell mitogen. For example, treatment with 200 μg/mL of OFS extract significantly ($p < 0.01$) reduced the tritium uptake of splenocytes by 26.4% compared to the control Con A treatment (158 001 cpm). These findings suggest that OFS extract inhibits mitogen-mediated DNA synthesis in T lymphocytes rather than in B lymphocytes.

Total Phenolic Content. The total phenolic content of OFS ethanol extract was determined spectrophotometrically using the Folin−Ciocalteu method described in the Materials and Methods section (Table 2). As shown in the table, the OFS extract contained 180.3 mg/g total phenolic compounds, which was

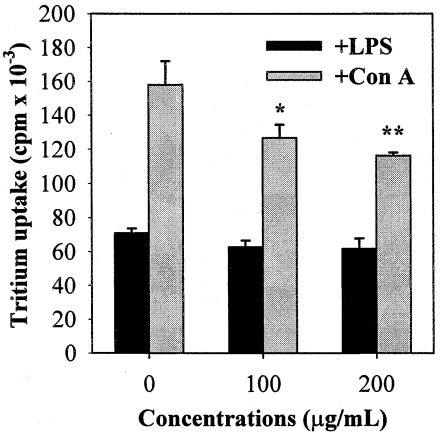

Figure 8. Effects of OFS extract on DNA synthesis in splenocytes. Splenocytes were stimulated with 5 μg/mL Con A and LPS for 48 h in the presence of OFS extract in the low serum condition and incubated in the presence of 1 μCi/mL [*methyl*-^{3}H] Thymidine for the last 12 h. Each bar represents the mean ± SE of triplicate experiments and the figure shows a representative result from three separate experiments. *p < 0.05 and **p < 0.01 indicate significant differences between the experimental and control values.

Table 2. Total Phenolic Content of the Ethanol Extract from OFS Stem[a]

sample	total phenolics[c] (mg/g)
origin preparation of OFS stem[b]	3.7 ± 0.7
ethanol extract of OFS stem	180.3 ± 18.6

[a] Experiments were performed according to the Folin–Ciocalteu method, as described in the Materials and Methods section. [b] The sample refers to freeze-dried OFS before it was extracted with ethanol. [c] Total phenolics are expressed as GAE; each experiment was performed in triplicate and the results are the mean ± SE.

much higher than that of the crude OFS preparation (3.7 mg/g). These results suggest that the greater antioxidant activity of OFS ethanol extract, compared to the crude OFS preparation, at the same concentration, is due to phenolic compounds.

DISCUSSION

We initially used an Fe^{3+}-dependent system to test the scavenging activity of OFS ethanol extract on radicals generated by iron, because hydroxyl radicals are known to be the most reactive of all the reduced forms of dioxygen and are thought to initiate cell damage in vivo (*25*). The results of ammonium thiocyanate and DNA nicking experiments showed that the OFS extract was an active scavenger of hydroxyl radicals, such that linoleic acid oxidation and DNA nicking were significantly prevented by the presence of OFS ethanol extract (**Figures 1** and **2**).

Next, we used a deoxyribose assay system to confirm the antioxidant activity of OFS ethanol extract, and found that the extract strongly inhibited hydroxyl radical-induced deoxyribose degradation in both site-specific and non-site-specific assays (**Figure 3**). In particular, the OFS extract inhibited deoxyribose degradation by chelating iron ions rather than by scavenging hydroxyl radicals directly. The indirect evidence of the scavenging activity of OFS extract on Fe^{3+}-dependent hydroxyl-radical generation was further confirmed using a direct approach with DPPH radicals, a stable radical used to evaluate the antioxidant activity of plant and microbial extracts (*26*, *27*). In this assay, the OFS ethanol extract exhibited powerful DPPH radical scavenging activity and the activity was similar to that of ascorbic acid (**Figure 4**), suggesting that OFS extract is a powerful natural antioxidant.

The possible pro-oxidant activity of OFS extract was also assessed using deoxyribose assays, because it might become biologically available to catalyze a free radical reaction at sites of tissue injury, especially in advanced atherosclerotic lesions and chronic inflammation (*28*). However, its ability to reduce the Fe^{3+}−EDTA complex, which represents its ability to stimulate hydroxyl radical generation, was observed only when low concentrations of the extract were added. High concentrations of OFS extract did not show stimulatory activity (**Table 1**). These results suggest that the pro-oxidant activity of OFS extract is readily overwhelmed by the antioxidant potential of the extract. Furthermore, when we consider the fact that the iron ions that catalyze the free radical reaction are safely sequestered within the human body, the limited pro-oxidant activity of OFS extract is unlikely to impose a significant problem in vivo.

Superoxide anions are the most common free radicals in vivo and are generated in a variety of biological systems, either by auto-oxidation processes or by enzymes. The concentration of superoxide anions increases under conditions of oxygenative stress and related situations (*20*, *29*, *30*). Moreover, superoxide anions produce other kinds of cell-damaging free radicals and oxidizing agents (*31*). Therefore, we used the NBT assay system to test whether OFS extract scavenges superoxide anions. In the system, xanthine oxidase, which is one of the main enzymatic sources of ROS in vivo, generated superoxide radicals, which consequently reduced NBT to yield blue formazan. The active inhibition of the NBT reduction induced by hypoxanthine-xanthine oxidase by OFS extract (**Figure 5**) suggests that OFS ethanol extract is a potential scavenger of superoxide anions, as well as hydroxyl radicals.

These results proved the excellent antioxidant activity of OFS extract in cell-free ROS-generating systems. To further evaluate the antioxidant property of OFS extract in living cells, flow cytometry analysis was used (**Figure 6**). In this experiment, treating the cells with OFS extract clearly inhibited the mitigation of $DCFH_2$ oxidation signals, which represents ROS-generation within cells due to the G/GO system. Furthermore, when the degree to which OFS ethanol extract protected against radical-induced cell injury was tested in cells using the MTT method, OFS extract significantly (p < 0.01) prevented splenocytes from death caused by hydroxyl radicals (**Figure 7**). Collectively, these results suggest that OFS extract effectively scavenges ROS and protects cells from radical-mediated injury.

Several active compounds, both synthetic and naturally occurring, exhibit antiproliferative activity against various cancer cell lines (*32*, *33*) and these findings led us to postulate that OFS extract regulates cell proliferation. OFS extract inhibited DNA synthesis in mitogen-stimulated splenocytes in a dose-dependent manner, as expected, supporting our hypothesis (**Figure 8**). However, the trypan blue staining experiment showed that the viability of cultured splenocytes treated with 200 μg/mL OFS extract for 24 h was about 90% that of control splenocytes (data not shown). This led us to hypothesize that the inhibitory effect of OFS ethanol extract on DNA synthesis is due to cytostatic rather than cytotoxic effects, although the exact mechanism by which OFS extract inhibits DNA synthesis in splenocytes needs to be clarified through additional experiments.

Finally, we examined the phenolic compound content of OFS ethanol extract using the Folin−Ciocalteu assay and compared it with that of the crude OFS preparation, because phenolic compounds are commonly found in plants and are reported to have multiple biological effects, including antioxidant activity (*32*−*37*). As expected, the OFS ethanol extract has a much higher phenolic compound content than the crude OFS preparation, supporting our hypothesis (**Table 2**). This clearly explained the reason for the antioxidant activity of OFS ethanol extract at low concentrations and for the dose-dependent inhibition of DNA synthesis. In similar reports, the enrichment of phenolic compounds within plant extracts is correlated with their enhanced antioxidant activity (*15*, *38*). In addition, antioxidant activity and the phenolic content of OFS extract were higher than those of other Korean plant-derived foods such as ginger root, balloon-flower root, and spinach, suggesting that OFS could be used as an antioxidant-rich functional food (*11*, *13*, *39*). Moreover, total phenolic content of OFS ethanol extract was 7.78-fold higher than that (23.2 mg/1 g) of ethanol extract of the leaves from *Rumex crispus* L., which is a perennial wild plant and has been consumed as a vegetable and used as a Turkish folk medicine (*40*). Studies on the chemical composition of the plant-derived functional foods reported that *Opuntia ficus-indica* (L.) Mill., the main plant source for natural colorants, betalains, was found to be a potential antioxidant against ABTS free radicals (*41*, *42*). However, the cactus species used in the study was different from the OFS used in this study (*7*, *11*). Furthermore, considering the condition to prepare the OFS ethanol extract, we strongly assume that the antioxidant action of OFS was not evolved from a single compound such as betalains (betacyanins and betaxanthins) but from flavonoids, such as quercetin and myrisetin, and vitamins.

In summary, it is well understood that the generation of ROS beyond the capacity of a biological system to eliminate them gives rise to oxidative stress. This stress may play a role in several diseases, such as heart disease, degenerative neuronal disease, and cancers (*5*, *29*, *43*, *44*). Furthermore, many biochemical and clinical studies suggest that natural and synthetic antioxidant compounds are helpful in treating diseases mediated by oxidative stresses. Our study demonstrated that an ethanol extract from OFS stem has excellent antioxidant activities. Hence, it is worthwhile to investigate the potential effectiveness of OFS in preventing oxidative stress-mediated disease further. Consequently, studies examining the composition of the OFS ethanol extract and determining the specific compounds responsible for its antioxidant activity, using bioorganic chemistry, mass spectrometry, and NMR spectrometry, are in progress.

ABBREVIATIONS USED

Con A, Concanavalin A; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; GAE, gallic acid equivalents; G/GO, glucose/glucose oxidase; H_2O_2, hydrogen peroxide; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; NBT, nitro blue tetrazolium; $^{\bullet}OH$, hydroxyl radical; $O_2^{\bullet-}$, superoxide anion; OFS, *Opuntia ficus-indica* var. *saboten;* ROS, reactive oxygen species; SOD, superoxide dismutase.

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