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Antioxidative Activity of Cherry Tomato (*Lycopersicon lycopersicum* var. *cerasiforme*) Extracts and Protective Effect for H_2O_2-induced Inhibition of Gap Junction Intercellular Communication

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Abstract This study was performed to analyze various antioxidants, to evaluate the antioxidative activities, and to measure the protective effect for gap junction intercellular communication (GJIC) to assess the functional potency of the cherry tomato. The ascorbic acid, lycopene, and β-carotene were measured at 503.4±9.6, 39.7±1.5, and 7.4±0.3 mg/100 g d.w., and α-, β+γ-, δ-tocopherol contents were measured at 8.3±0.1, 1.7±0.0, and 0.1±0.0 mg/100 g d.w., respectively. Cherry tomato extract using hexane/acetone/EtOH (2:1:1, CTE) exhibited a ABTS radical scavenging activity with an IC_{50} value of 48.83±0.30 μg/mL. The cherry tomato protected against the inhibition of GJIC induced by H_2O_2 in WB-F344 rat liver epithelial cells, and the reduction in phosphorylated Cx43 was most clearly correlated with the concentration of CTE. These results demonstrated that the cherry tomato harbors a wealth of potent antioxidants and might be protect human body against the inhibition of the GJIC by toxic components.

Keywords: cherry tomato, antioxidant, lycopene, gap junction intercellular communication

Introduction

The cherry tomato (*Lycopersicon lycopersicum* var. *cerasiforme*) is a smaller variant of the general tomato, one of the most popular fruits worldwide, and contains relatively high levels of antioxidants, including carotenoids, tocopherols, flavonoids, ascorbic acid, etc (1,2). Consumption of the cherry tomato in Korea has increased continuously since 1990, because the fruit can be obtained in a fresh state without artificial processing, and because it is so convenient to obtain and consume. The cherry tomato is generally considered to be similar, but not identical to the wild precursor of the general tomato.

Generally, a great deal of evidence has accumulated regarding the tomato's beneficial effects, most notably its abilities to reduce or ameliorate cancer and cardiovascular diseases (3,4). Lycopene, in particular, has been demonstrated to have a profound antioxidant activity, exhibiting a high level of physical quenching activity with singlet oxygen and exhibiting chemopreventive activity for cancers (5). Additionally, 2 flavonols, quercetin, and kaempferol, appear to exert particularly strong cardiovascular protective effects (6). The cherry tomato and general tomato, especially, are remarkably different in terms of their β-carotene and vitamin C contents, which were 3 and 2 times higher, respectively, in the cherry tomato (7). Considering these differences, the health-improving effects of cherry tomato could be more profound than those of the general tomato. However, information regarding the functionality of the cherry tomato is currently lacking. Studies regarding the cherry tomato have generally focused on the improvement of quality and maintenance by the development of new cultivars (8), by new package, ozone treatment, and storage techniques (9), and by developing new methods of cultivation (10). Recently, a proteomic approach was utilized in a study of the fruit development of the cherry tomato (11).

In this study, we analyzed tocopherols, ascorbic acid, and carotenoids including lycopene and β-carotene as antioxidants from cherry tomatoes, and evaluated the antioxidative activities of various cherry tomato extracts (CTE), using different solvents. We then assessed the cytotoxicity of CTE and the protective effect for gap junction intercellular communication in order to assess the potency of the functional values of the cherry tomato.

Materials and Methods

Sample preparation and extracts The cherry tomato samples were kindly provided by Buyeo Nonghyup Agricultural District Distribution (Chungnam, Korea). The cherry tomatoes were sorted, washed, and dried with a freeze-dryer, then stored at −70°C until use. In order to compare the effects of various organic solvents, the samples (5 g) were extracted with various solvents (50 mL) for 2 hr at room temperature; 80% ethanol (EtOH), 80% methanol (MeOH), 80% acetone, and hexane:acetone:EtOH (2:1:1, v/v/v; HAE). All extracts were utilized to assess the antioxidative activity. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

High performance liquid chromatography (HPLC) analysis of tocopherol, ascorbic acid, and carotenoids The analyses were conducted using a Jasco HPLC system (Tokyo, Japan) comprised of a quaternary pump, a versatile autosampler, and a column oven. For tocopherol analysis, the dried tomato samples (0.5 g) were extracted with

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methanol (5 mL) for 30 min. The mixture was then centrifuged and the supernatant was filtered through a 0.45-mm Millipore filter (PVDF, Whatman, Clifton, NJ, USA). The HPLC analysis of α-, β-, γ-, and δ-tocopherol was conducted with a mobile phase consisting of methanol, acetonitrile, isopropanol, and 1% acetic acid. The detector (Jasco FP-2020) was set at an excitation wavelength of 298 nm and an emission wavelength of 328 nm. After injecting 20 μL of sample, separation was conducted on a Nova-pack C18 150×3.9 mm column (Waters, Santa Clara, CA, USA).

For vitamin C analysis, dried tomato sample (0.5 g) was added to 5% metaphosphoric acid (50 mM) and mixed with a vortex mixer. The mixture was then centrifuged for 5 min at 3,000×g. Twenty μL of supernatants was injected into an HPLC system for analysis. The mobile phase was acetonitrile and 50 mM metaphosphoric acid (7:3, v/v) and the ultraviolet (UV) detector (JascoUV-2075) was set at 254 nm. The samples were separated using an YMC-Pack Polyamine 250×4.6 mm column (YMC Co., Ltd., Kyoto, Japan).

For carotenoid analysis, the dried tomato sample (0.5 g) was treated with 15% KOH and 10% phrogaroll, then mixed with a vortex mixer. The mixture was boiled for 30 min at 60°C under a reflux condenser. Extraction was conducted using petroleum ether and mixed by inversion. Organic layers were collected 4 times, and the solvent was evaporated to dryness. The dried sample was then reconstituted with isopropanol and chloroforms (1:1, v/v) and thoroughly mixed. Twenty μL of supernatants were injected. The HPLC separation of various carotenoid standards was conducted with a mobile phase consisting of solvents A, B, and C in a gradient, in which A was methanol, methyl tertiary-butyl ether (MTBE), and water (81:15:4, v/v/v), B was methanol and MTBE (1:9, v/v), and C was 70% methanol. The gradient elution was filtered through a 0.45-mm Millipore filter (PVDF) and degassed prior to use. The flow rate was set at 1.0 mL/min. The detector (Jasco UV-2075 and FP-2020) was set at 450 nm. The samples were separated using an YMC-Carotenoid 250×4.6 mm column.

Radical scavenging activity 1,1-Diphenyl-2-picrylhydrazy (DPPH: Sigma-Aldrich) radical scavenging activity was assessed in accordance with the method described by Abe *et al*. (12). Each extract was diluted to several concentrations, mixed with 4×10^4 M DPPH, then vortexed for 10 sec. The samples were set aside for 15 min, and the absorbance was measured at 525 nm. MeOH without DPPH radicals was used as a blank reference. Absorbance was converted to DPPH radical scavenging activity in accordance with the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

ABTS radical scavenging activity was evaluated in accordance with the method developed by Arnao *et al*. (13) with some modifications. In brief, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 2,2-azobis(2-amidino-propane) dihydrochloride (AAPH) were dissolved into 100 mM potassium phosphate buffer (pH 7.4) including 150 mM NaCl, respectively, then mixed with an identical amount (ca. 150 mL) at 68°C to generate $ABTS^{\bullet+}$. The absorbance of the reactant was later adjusted to 0.650±0.02 at ambient temperature at a wavelength of 734 nm. A 980 μL of each extract and 20 μL of MeOH were mixed with $ABTS^{\bullet+}$ solution and allowed to react for 10 min at 37°C. ABTS radical scavenging activity was calculated by the change in absorbance.

Cell culture WB-F344 rat liver epithelial cells (WB cells) were generously provided by Prof. Kang KS (Seoul National University, Seoul, Korea). The cells were cultured in D-media (Formula No. 78-5470EF; Gibco BRL, Grand Island, NY, USA) supplemented with 5%(v/v) fetal bovine serum (FBS, Gibco BRL) and penicillin/streptomycin at 37°C in a humidified oven in an atmosphere of 5% CO_2 and 95% air. Sample treatment for each analysis was conducted using D-media without FBS. The cells were grown in 75-mm tissue culture plates and the culture medium was changed every other day.

Cytotoxicity Cytotoxicity was assessed via MTT assay (14). The cells were cultured in 24-well plates at 5×10^4 cells/well in D-media with 5% FBS for 24 hr. Each well was then filled with fresh D-media without FBS containing various doses of HAE extracts, and the cells were incubated for an additional 24 hr at 37°C. Each well was then incubated for 4 hr with MTT. The liquid was removed and dimethyl sulfuroxide (DMSO) was added in order to dissolve the solid residue. The optical density of each well was then determined at 570 nm using a microplate reader (EMax Precision; Molecular Devices, Sunnyvale, CA, USA). Cytotoxicity was evaluated by the absorbance reduction of the samples at various concentrations (10, 25, 50, 100, 150, and 200 μg/ mL) with or without catalase (Sigma-Aldrich, 100 units/mL), as compared with the untreated control samples.

Gap junctional intercellular communication (GJIC) bioassay GJIC was measured via the scrape-loading/dye-transfer technique (SL/DT) (15). WB cells were treated with various concentrations of HAE extract for 24 hr. The GJIC assay was conducted with non-cytotoxic doses of HAE extract, as determined by MTT assay. After incubation, the cells were treated for 1 hr with 500 μM H_2O_2 and then washed twice with 2 mL of phosphate buffered saline (PBS). Lucifer yellow was added to the washed cells, and three scrapings were made with a surgical steel scalpel at low light intensity. Each scraping was performed so as to traverse a large group of confluent cells. After a 3 min incubation period, the cells were washed 4 times with 2 mL PBS and then fixed with 2 mL of a 4% formalin solution. The numbers of communicating cells, as indicated by the dye-receiving cells on the line perpendicular to the scrape-line, were counted under an inverted fluorescence microscope (Ix70; Olympus, Okaya, Japan).

Western blot analysis After the SL/DT analysis, the cells (5×10^4 cells/well in a 100-mm dish) were collected and washed in PBS. After centrifugation, cell lysis was conducted at 4°C by 15 min of vigorous shaking in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH

Table 1. Contents of ascorbic acid, tocopherols, and carotenoids from cherry tomatoes (mg/100 g d.w.)

Ascorbic acid	Tocopherol			Carotenoids	
	α	β+δ	δ	Lycopene	β-Carotene
503.4±9.6	8.3±0.1	1.7±0.0	0.1±0.0	39.7±1.5	7.4±0.3

7.4), 50 mM β-glycerophosphate, 20 mM NaF, 20 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, and protease inhibitors]. After 15 min of centrifugation at 13,000×g, the supernatant was separated and stored at -70°C until use. Protein content was determined using Bradford reagent (Bio-Rad Co., Hercules, CA, USA) and the supernatants were separated via 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) (16). Western blot analysis of Cx43 was conducted, and the proteins were transferred to PVDF membranes at 100 V, 350 mA for 2 hr. Antibody was utilized in accordance with the manufacturer's instructions and the protein bands were detected with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistics All samples were based on dry weight. Quantitative data are expressed as the mean±standard deviation (SD) of at least 3 replicate determinations. Each experimental set was compared with one-way analysis of variance (ANOVA) and Duncan's multiple-range test ($p<0.05$) using SAS program (SAS Institute, Cary, NC, USA).

Results and Discussion

Antioxidant contents and antioxidative activity Increased consumption of cherry tomatoes could perform a crucial function in improving protection against free radicals in the human body, and the cherry tomato is generally regarded as a fruit with potent health-improving activities. In our study, we assessed the contents of ascorbic acid, tocopherol analogues, and carotenoids, all of which are representative antioxidants, prior to assessing antioxidative activity.

The ascorbic acid content of the cherry tomato was measured at 503.4±9.6 mg/100 g d.w. shown in Table 1, which is higher than in other reports. As compared with 14 different cherry tomato cultivars, cv. Corbus evidenced the highest ascorbic acid content, at 210 mg/100 g d.w., and the 'Salentino', 'Lycorino', and 'Sakura' varieties evidenced a low content (<33 mg/kg of fresh weight) (2). The ascorbic acid content of the cherry tomato in the Korean Food Composition Table (7) was reported as 21 mg/100 g edible portion (approx. 380 mg/100 g d.w.). This means that the cherry tomatoes used in this experiment contained quite a substantial quantity of ascorbic acid.

Tocopherols having vitamin E activity were classified into α-, β-, γ-, and δ-tocopherol, and α-tocopherol is the most active form of vitamin E, which is essential to improving human health (17). In this experiment, we identified α-, β+γ-, and δ-tocopherol via HPLC, and those values were measured to be 8.3±0.1, 1.7±0.0, and 0.1±0.0 mg/100 g d.w., respectively (Table 1). Recently, the α-tocopherol contents of 14 cherry tomato cultivars were reported to be in the range of 2.5-11.3 mg/100 g d.w. (2). Our results demonstrated that α-tocopherol content was the highest of all of them, and reached 80% of the total tocopherol content.

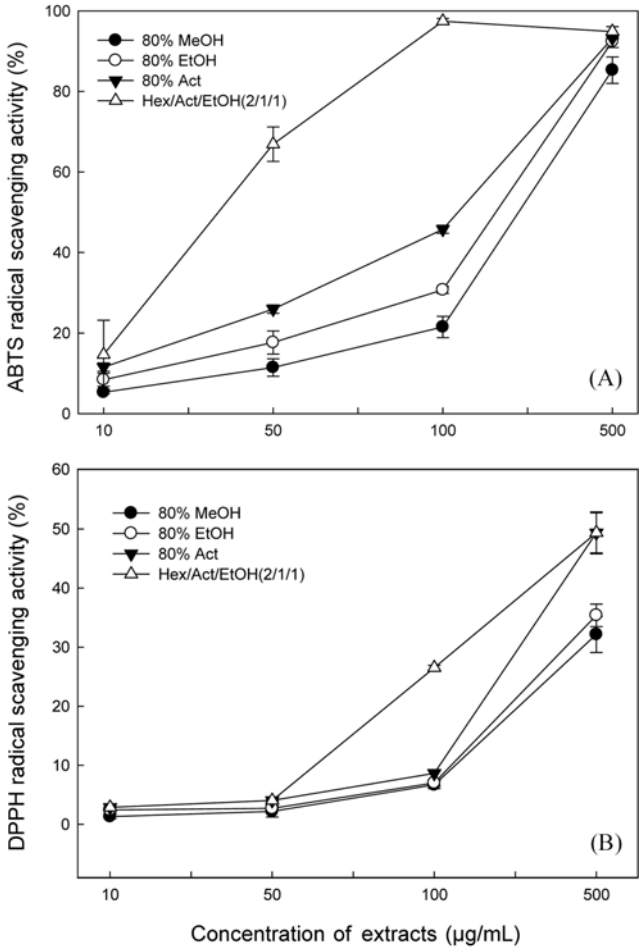

Fig. 1. ABTS and DPPH radical scavenging activities of various cherry tomato extracts in different solvent systems.

The lycopene content was 39.7±1.5 mg/100 g d.w. (Table 1), which was considerably lower than other reported results. Lenucci *et al.* (2), in a comparative study of 14 cherry tomato cultivars, asserted that lycopene contents evidenced profound variation, in a range between 50-130 mg/100 g d.w. Lycopene contents were very diverse, and varied by harvesting conditions as well as by cultivar. Takeoka *et al.* (8) confirmed that field-grown tomatoes generally harbored higher levels of lycopene than greenhouse-grown tomatoes. β-Carotene content was 7.4±0.3 mg/100 g d.w. and the ratio of β-carotene to lycopene was 15:85. β-carotene accounted for 4.3-12.2% of the total carotenoids in 14 cherry tomato cultivars (2). Our results demonstrated that the ratio of β-carotene was much higher than this.

The antioxidant activity of the cherry tomato was compared using different solvent systems, via measurements of

Table 2. IC_{50} values of cherry tomato extracts using various solvents in DPPH and ABTS radical scavenging activities

(μg/mL)

	ABTS radical scavenging activities	DPPH radical scavenging activities
80% MeOH	249.24±6.51	256.96±8.46
80% EtOH	226.64±3.28	267.03±3.87
80% Acetone	155.60±2.43	271. 69±0.01
HAE[1)]	48.83±0.30	213.67±3.56

[1)]Hexane:acetone:ethanol=2:1:1 (v:v:v).

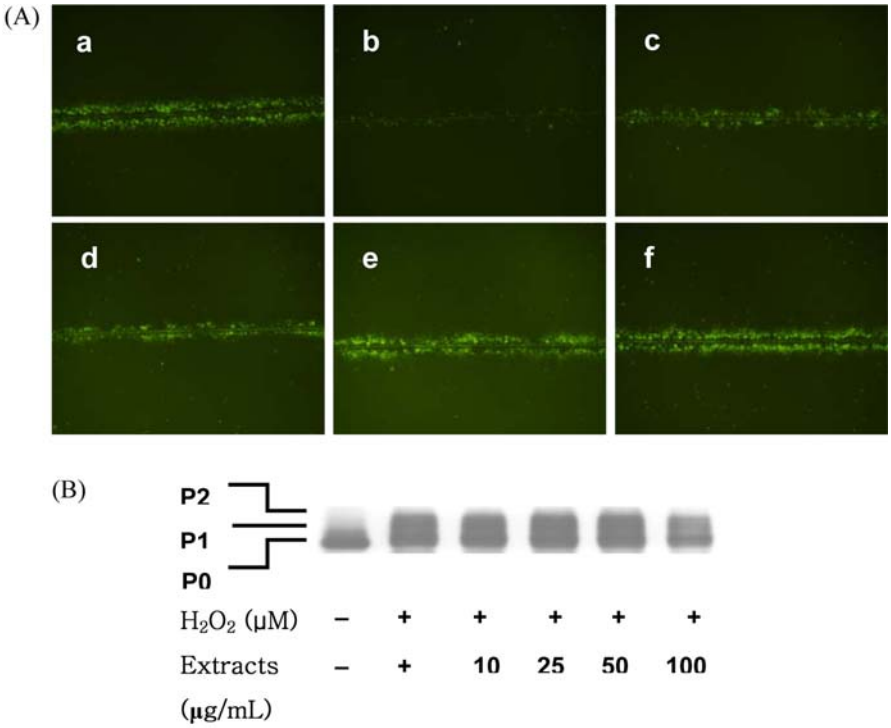

Fig. 2. Effects of hexane:acetone:EtOH (2/1/1, v/v/v) extract from cherry tomato (CTE) on the inhibition of GJIC induced by H_2O_2 in WB-F344 cells (A) and Western blot analysis on the changes by CTE in the phosphorylation pattern of Cx43 (B). (a) Treated without CTE and 500 μM H_2O_2, (b) 0, (c) 10, (d) 25, (e) 50, and (f) 100 μg/mL CTE treated with 500 μM H_2O_2.

ABTS and DPPH radical scavenging activity. CTEs in all solvent systems exhibited radical scavenging activities in a dose-dependent manner when measured via both DPPH and ABTS techniques (Fig. 1). In particular, the HAE extracts evidenced a ABTS radical scavenging activity with an IC_{50} value of 48.83±0.30 μg/mL (Table 2) comparing other solvent extracts which is approximately 4 to 5 times as active as other solvent extracts (Fig. 1A). DPPH radical scavenging activities in various CTEs were similar up to 50 μg/mL; however, that value increased to 100 μg/mL in the HAE extracts (Fig. 1B) and the IC_{50} value was 213.67± 3.56 μg/mL (Table 2). According to the reports of Scalfi *et al*. (18) and George *et al*. (19), cherry tomatoes evidence antioxidant power significantly more pronounced than that of general tomatoes, and this is probably attributable to the cherry tomato's higher ascorbic acid, tocopherol, and lycopene contents (19-21).

In this study, we demonstrated that the cherry tomatoes examined in this experiment harbor abundant ascorbic acid, tocopherol, and carotenoids. In particular, ascorbic acid content was extremely high. This indicates that the cherry tomato harbors both hydrophilic and lipophilic antioxidants, and this might contribute to the profound antioxidative activity observed in the HAE extracts.

Cytotoxicity and protective effects for GJIC We screened the proper concentration evidenced no cytotoxic effects, because the monolayer density can affect communication between cells. We evaluated the potential cytotoxic effects of CTE at a range between 10-200 μg/mL, and detected no cytotoxic effects in the cells that were treated for 1 hr (p>0.05), and we conducted the following experiments with less than 200 μg/mL CTE.

Gap junction channels, structures in the plasma membrane used for cell-to-cell communication, perform crucial functions in the maintenance of the homeostatic regulation of cell growth and differentiation, as well as a host of other physiological processes (16,22). Consequently, damage to the gap junction as the result of the reduction of numbers and size caused abnormal cell proliferation (23-25), and this phenomenon was correlated with the upregulation of hyper-phosphorylated connexin 43 (26).

In this study, we evaluated the protective effects of CTE for the inhibition of H_2O_2-induced GJIC in highly communicating WB-F344 rat liver epithelial cells, which express connexin43 as their predominant gap junction protein (27,28). As shown in Fig. 2A, the gap junction in normal cells (a) was decreased significantly about 83% by H_2O_2 treatment, and was increased in a dose-dependent manner by CTE treatment. Specifically, the recovery rates at 50 and 100 μg/mL were 63.6 and 69.1%, respectively, and this indicated that CTE was very effective in facilitating the recovery of the gap junction. In order to verify these protective effects on the basis of a functional mechanism, we assessed the phosphorylation pattern of Cx43 via Western blot analysis. The Cx43-P_1 and -P_2 bands represent the hyperphosphorylated form of Cx43, and an increase in the phosphorylated form was correlated with the inhibition of the gap junction (29). In this study, H_2O_2 treatment shifted unphosphorylated Cx43 band (P0) to the phosphorylation bands (P1 and P2) shown in Fig. 2B, and those bands were decreased as the result of CTE treatments. Increases in the recovery rate of GJIC in SL/DT techniques and the reduction in phosphorylated Cx43 were most clearly correlated at the concentration of 100 μg/mL CTE. These results indicated that CTE effectively recovered the GJIC inhibited by the tumor promoter. This must be associated with the active components from CTE. Tocopherol analogues may exert adverse effects on gap junctional intercellular communication (GJIC), in addition to antioxidant properties (30). The lycopene oxidation product was also shown to enhance gap junctional communication (31), and this result indicated that the biological metabolites of lycopene in the human body would prove to be potent active components.

In conclusion, this study demonstrated that the cherry tomato harbors a wealth of potent antioxidants, including ascorbic acid and α-tocopherol as well as lycopene and β-carotene, and might be protect human body against the inhibition of the gap junctional intercellular communication by toxic components.

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