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RESEARCH ARTICLE

Evidence for Protective Effects of Coffees on Oxidative Stress-induced Apoptosis through Antioxidant Capacity of Phenolics

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Abstract This study evaluated total phenolics, total flavonoids, and antioxidant capacity of randomly selected regular and decaffeinated coffees commercially available in Korea and their protective effects in human hepatic epithelial HepG2 cell line against oxidative stress. All coffees tested exhibited potent antioxidant capacity in chemical systems and, consequently, significant protection of cells from oxidative stress *in vitro* in a dose-dependent manner. In particular, H_2O_2-induced apoptosis as evaluated by annexin V staining and flow cytometry was prevented by coffee extracts, resulting in the enhanced cell viability. Of interest, the content of total phenolics and flavonoids in coffees demonstrated a positive correlation with antioxidant capacity, indicating that the antioxidant capacity of coffees may be attributed to those phytochemicals. In accordance with previous studies, caffeoylquinic acid (CQA) and its derivatives including 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA were identified as phenolic phytochemicals by a reversed-phase HPLC, with 5-CQA being a major component. Taken together, the present study demonstrated protective effects of regular and decaffeinated coffees on cells *in vitro* against overwhelming oxidative stress due to richness in phenolics, especially CQA and its derivatives. Coffees, regular or decaffeinated, may serve as a good source of health-beneficial phytochemicals in diet.

Keywords: anti-apoptosis, caffeoylquinic acid, flow cytometry, total phenolics, vitamin C equivalent

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Introduction

As a consequence of cellular oxygen metabolism, reactive oxygen species (ROS) are constantly generated and contribute to a variety of physiological activities including host defense and cellular signaling, even though excessive production of ROS evokes detrimental oxidative stress (1,2). In order to prevent possible damage to biological molecules by oxidative stress, aerobic organisms possess well-integrated *in vivo* defense systems, including enzymes (superoxide dismutase, catalase, and glutathione peroxidase), metal-chelating macromolecules (albumin, transferrin, and lactoferrin), and an array of radical scavenging antioxidants (vitamin C, uric acid, vitamin E, carotenoids, and phenolics). Phenolics are ubiquitous constituents of higher plants and exist in a wide range of plant-based beverages such as juice, wine, tea, and coffee. Many phenolics have potential antioxidant properties due in part to their characteristic chemical structures, which may aid in preventing degenerative diseases such as cancer, cardiovascular diseases, and neurodegenerative diseases against oxidative stress (3).

Coffee is one of the most popular drinks in the world. More than 500 billion cups of coffee are consumed annually worldwide, with the number being continuously increasing, partly due to its characteristic taste and flavor. Recent studies have revealed the beneficial effects of coffee on health with considerable evidence supporting the

benefits of moderate coffee consumption (4,5). In coffee, chlorogenic acid and its derivatives among phenolics are present predominantly as a family of esters linking certain hydroxycinnamic acids and quinic acid (6). Chlorogenic acids play an important role in the formation of coffee flavor, thereby considerably contributing to coffee quality (7). Chlorogenic acids also possessed their health-beneficial properties, which may be explained by their potent antioxidant capacity (8,9).

Antioxidant capacity of coffee has been evaluated using various chemical methods such as ABTS, FRAP, and oxygen radical absorbance capacity (ORAC) assays (10-13). The antioxidant capacity of coffee was mainly expressed in μmole of Trolox equivalents (10-13), where Trolox is a water-soluble synthetic vitamin E analogue. With vitamin C being a naturally occurring and major antioxidative compound, however, little information on antioxidant capacity of coffee in terms of vitamin C equivalents is available. The purposes of this study were to quantitatively determine total phenolics, total flavonoids, and antioxidant capacity of commercially available coffee products and to identify caffeoylquinic acid (chlorogenic acid) and its derivatives as major coffee phenolics by a reversed-phase HPLC system. Furthermore, the protective effects of coffee phenolics against oxidative stress in human hepatic carcinoma cell line, HepG2 cells, were evaluated.

Materials and Methods

Samples Four brands of roasted coffees were randomly purchased in a local market in Korea. Two brands of coffees were regular and 2 were decaffeinated (Table 1). The type, origin, and species of coffees used in this study are presented in Table 1.

Chemicals Folin-Ciocalteu's phenol reagent, ABTS, AAPH, DPPH, gallic acid, catechin, ascorbic acid, and *in vitro* toxicology assay kit of lactate dehydrogenase (LDH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MEM medium was purchased from Welgene (Daegu, Korea). Fetal bovine serum, streptomycin, and penicillin were obtained from Gibco BRL (Grand Island, NY, USA). All other chemicals used were of analytical or HPLC grade.

Sample preparation Roasted whole coffee beans were ground prior to brew preparation. Ground coffee (20 g) was brewed by a commercial automatic drip coffee maker (Philips HD-7466; Koninklijke Philips Electronics N.V., Amsterdam, Netherlands) with 450 mL of deionized distilled water (DDW). The brewed coffee was evaporated by using a rotary evaporator under reduced pressure at 40ºC. The completely dried coffee extracts were kept at 20ºC until analysis.

Determination of total phenolics The contents of total phenolics in coffee were measured by a colorimetric assay using Folin-Ciocalteu's phenol reagent (14). An aliquot (0.2 mL) of the appropriately diluted sample was mixed with 2.6 mL of DDW. At zero-time, 0.2 mL of Folin-Ciocalteu's phenol reagent was added to the mixture. After 6 min, 2 mL of 7%(w/v) Na_2CO_3 solution was added. The absorbance of the mixture was measured at 750 nm at 90 min. The content of total phenolics in coffee was expressed as mg gallic acid equivalents (GAE)/g coffee.

Determination of total flavonoids The content of total flavonoids in coffee was measured using a modified method of Jia *et al.* (15). Five-hundred μL of coffee extract was mixed with 3.2 mL of DDW. Then 150 μL of 5%(w/v) $NaNO_2$ was added to the mixture. At 5 min, 150 μL of 10%(w/v) $AlCl_3$ was added. At 6 min, 1 mL of 1 M NaOH was added. Absorbance of the mixture was measured immediately at 510 nm. The content of total flavonoids in coffee was presented as mg catechin equivalents (CE)/g coffee.

Determination of ABTS radical scavenging capacity Antioxidant capacity was measured by using blue/green ABTS radicals described in literature (16). The solution of ABTS radicals was adjusted to absorbance of 0.650±0.020 at 734 nm. The reaction between ABTS radicals and sample was done at 37ºC for 10 min. Antioxidant capacity of coffee was expressed as mg vitamin C equivalents (VCE)/g coffee.

Determination of DPPH radical scavenging capacity Antioxidant capacity of coffee was determined by using DPPH radical chromogens (16). The absorbance of fresh DPPH radicals in 80%(v/v) aqueous methanol was set at 0.650±0.020 at 517 nm. The reaction between DPPH radicals and sample was allowed at 23ºC for 30 min. The antioxidant capacity of coffee was expressed as mg VCE/g coffee.

Determination of ORAC The ORAC assay was performed to determine antioxidant capacity of coffee using a fluorescence detector (17). The ORAC assay is based on a measurement of the oxidative decomposition of fluorescein following mixing and heating with a free radical generator (AAPH); the more oxidative decomposition of fluorescein, the less fluorescent intensity. One-hundred μL of fluorescein solution (81.6 nM) was mixed with antioxidant standard (vitamin C) or coffee extract in a 96-

well plate at 37°C. At 10 min, 25 μL of AAPH (153 mM) was added. Fluorescence was detected every minute for 90 min using a fluorometer (Tecan infinite M200; Tecan Sytstems Inc., San Jose, CA, USA) at 485 nm for excitation and 520 nm for emission. The regression equation was obtained between vitamin C concentration and the net area under the cover. Antioxidant capacity measured by ORAC assay was expressed as mg VCE/g coffee.

Cell culture Human hepatic epithelial HepG2 cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were cultured with MEM medium containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in an incubator with 5% CO_2 at 37°C.

Determination of cell viability Sample cytotoxicity and cell viability were determined by MTT assay, based on the reducing activity of cellular reductase in mitochondria of viable cells. HepG2 cells (1×10^4/well) were seeded in a 96-well plate and incubated in a humidified incubator with 5% CO_2 at 37°C for 24 h. After removing the medium, the cells were treated with serum-free medium containing various concentrations of the samples. Following 24 h incubation, the cells were washed with phosphate buffered saline (PBS) followed by either cytotoxicity or protection assay. For the cytotoxicity assay, cells were treated with MTT reagent for 3 h. MTT formazan was dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured using aforementioned fluorometer at 570 and 630 nm for test and reference wavelength, respectively. For the protection assay, HepG2 cells were stressed in the presence of 500 μM of H_2O_2 for 3 h, followed by the MTT assay as described.

Measurement of cell membrane integrity Damaged cell membrane was evaluated by measuring LDH released into the medium. Oxidative stress was caused by 500 μM of H_2O_2. The amount of intracellular LDH release was determined using the *in vitro* toxicology assay kit. The absorbance was measured at 490 and 690 nm for test and reference wavelength, respectively.

Measurement of intracellular oxidative stress The level of intracellular ROS was determined by fluorescent assay using DCFH-DA (18). HepG2 cells were seeded at a density of 1×10^4 cells/well on 96-well plates in MEM medium and incubated for 24 h. Then, cells were treated with various concentrations of coffee extracts for 24 h. After 30 min incubation with 50 μM DCFH-DA in PBS, cells were treated with 300 μM of H_2O_2 for 3 h. The fluorescence was measured at 485 nm for excitation and 530 nm for emission.

Analysis of flow cytometry By double staining of FITC annexin V and propidium iodide (PI), cell apoptosis and necrosis were identified (19). In this regard, cells were incubated for 24 h and pretreated for 24 h with various concentrations of coffee extracts. After treatment of 500 μM of H_2O_2 as oxidative stress to HepG2 cells for 2 h, cells were stained by using FITC annexin V apoptosis detection kit I (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Within 1 h, cells were analyzed by flow cytometry (FACS Aria II; BD Biosciences).

HPLC analysis of coffee phenolics Caffeoylquinic acid (CQA) and its derivatives were quantitatively analyzed by using an HPLC system (LC20; Shimadzu, Kyoto, Japan) equipped with autosampler, vacuum degasser, binary pump, and photodiode array detector. A reversed-phase analytical column used was a C18 Symmetry column (5 μm×4.6 mm ×250 mm; Waters, Milford, MA, USA). Injection volume was 20 μL. The flow rate was kept at 1.0 mL/min. The detector was set at 320 nm. The solvent gradient conditions of binary mobile phases (solvent A, 0.1%(v/v) formic acid in DDW; solvent B, 0.1%(v/v) formic acid in acetonitrile) were as follows: 92% A/8% B at 0 min, 89% A/11% B at 4 min, 87% A/13% B at 20 min, 80% A/20% B at 27.5 min, 40% A/60% B at 50 min, 92% A/8% B at 52 min, and 92% A/8% B at 60 min. Coffee phenolics were tentatively identified by comparing UV-visible spectra, retention times, and spike-input of authentic standards.

Statistical analysis All experiments were performed in triplicate. Statistical analyses were performed using a dedicated software package (SAS 9.0; SAS Institute, Inc., Cary, NC, USA). Significant differences were verified by Duncan's multiple range tests at 95% confidence level.

Results and Discussion

Extraction yield The amount of extracted solids from approximately 20 g of roasted coffees ranged from 4.5 to 4.7 g (Table 1). Extraction yields ranged from 22.5 to 23.5%, which were not significantly different (Table 1).

Total phenolics and total flavonoids The contents of total phenolics ranged from 25.21 to 28.30 mg GAE/g (Table 2). The contents of total phenolics of coffee tested were in decreasing orders as follows: brand D (decaffeinated) > brand A (regular) > brand B (regular) > brand C (decaffeinated). In a recent study, the contents of total phenolics were at 21.01 to 42.37 mg GAE/g coffee, where these contents were significantly affected by coffee

Table 1. Characteristics and extraction yield of regular and decaffeinated coffees commercially available in Korea

Coffee	Type	Origin	Species	Amount brewed (g)	Final volume brewed (mL)	Extracted solid (g)	Yield (%)
Regular							
Brand A	whole bean	Indonesia	Arabica	20.03±0.02[a1)]	400±5[a]	4.5±0.3[a]	22.5±1.5[a]
Brand B	ground	NA[2)]	Arabica	20.03±0.04[a]	403±2[a]	4.7±0.2[a]	23.5±1.0[a]
Decaffeinated							
Brand C	whole bean	Latin America	Arabica	20.05±0.05[a]	395±5[a]	4.6±0.1[a]	22.9±0.5[a]
Brand D	whole bean	Indonesia	Arabica	20.04±0.03[a]	395±10[a]	4.5±0.1[a]	22.5±0.5[a]

[1)]Data are presented as mean±SD (n=3); Different superscripts in the same column show the significant difference by Duncan's multiple range test (p<0.05).
[2)]NA, not available

Table 2. Total phenolics, total flavonoids, and antioxidant capacity of regular and decaffeinated coffees commercially available in Korea

Coffee	Total phenolics (mg GAE[1)]/g)	Total flavonoids (mg CE[2)]/g)	Antioxidant capacity (mg VCE[3)]/g)		
			ABTS	DPPH	ORAC
Regular					
Brand A	26.29±0.49[b4)]	14.08±0.32[b]	26.12±0.18[b]	19.59±1.39[b]	24.81±3.83[a]
Brand B	26.06±0.14[b]	15.07±0.43[a]	25.12±0.37[c]	18.95±1.05[bc]	25.65±3.55[a]
Decaffeinated					
Brand C	25.21±0.54[c]	12.77±0.11[c]	23.19±0.18[d]	18.14±1.19[c]	20.09±4.98[a]
Brand D	28.30±0.58[a]	15.26±0.57[a]	28.45±0.61[a]	21.24±2.19[a]	25.25±3.28[a]

[1)]GAE, gallic acid equivalents
[2)]CE, catechin equivalents
[3)]VCE, vitamin C equivalents
[4)]Data are presented as mean±SD (n=3); Different superscripts in the same column show the significant difference by Duncan's multiple range test (p<0.05).

cultivars rather than by the roasting degrees (20). It has been reported, however, that coffee contains a relatively lower level of total phenolics at 161 mg GAE/cup of 200 mL, which is equivalent to 13.4 mg GAE/g roasted and ground coffee (8) and at 2.5 to 4.3 mg GAE/g coffee (21). This discrepancy in total phenolics reported in the literature may result from varieties, brands, brewing methods, roasting degrees, and geographical origins (13,20-22).

The contents of total flavonoids ranged from 12.77 to 15.26 mg CE/g (Table 2). The contents of total flavonoids of coffee tested were in decreasing orders as follows: brand D (decaffeinated) > brand B (regular) > brand A (regular) > brand C (decaffeinated). Total flavonoids had a linear correlation (r^2=0.532) with total phenolics (data not shown). The total flavonoids have been found to account for approximately 44% of total phenolics in coffees from *Coffea arabica* and *C. canephora* (20).

Antioxidant capacity Three different methods (ABTS, DPPH, and ORAC) were used to evaluate the antioxidant capacity which was expressed in vitamin C equivalents (VCE) in regular and decaffeinated coffees (Table 2). Antioxidant capacity evaluated by using ABTS radicals ranged from 23.19 to 28.45 mg VCE/g. Antioxidant capacity in the ABTS assay decreased as follows: brand D (decaffeinated) > brand A (regular) > brand B (regular) > brand C (decaffeinated).

DPPH radical is usually used to evaluate the antioxidant capacity of foods. This method is based on the reduction of DPPH radical to non-radical form DPPH-H in the presence of a hydrogen-donating antioxidant (23). The antioxidant capacity of coffee tested ranged from 18.14 to 21.24 mg VCE/g (Table 2). In the DPPH assay, antioxidant capacity of coffees tested decreased as follows: brand D (decaffeinated) > brand A (regular) > brand B (regular) > brand C (decaffeinated).

The ORAC assay is a simple, sensitive, and reliable method to measure the peroxyl radical absorbing capacity of antioxidants with a relatively small sample volume of a few μL (24). The antioxidant capacity of coffees determined by the ORAC assay exhibited at 20.09 to 25.65 mg VCE/g, which was not significantly different among the coffees tested (Table 2). In the ORAC assay, the antioxidant capacity of coffees tested decreased as follows: brand B (regular) > brand D (decaffeinated) > brand A (regular) > brand C (decaffeinated).

Arabica and Robusta are usually blended for the production of coffee with balanced flavor and taste. Similar antioxidant capacity assessed by the *in vitro* LDL oxidation model was demonstrated between Arabica and Robusta

coffees (25). Therefore, blending of these coffee beans may minimally influence antioxidant capacity of final products. Decaffeinated coffee exhibited similar antioxidant capacity to regular coffee (25). Decaffeinated coffee brand D had the highest antioxidant capacity in the ABTS and DPPH assays, whereas regular coffee brand B was highest in the ORAC assay (Table 2). No significant difference of antioxidant capacity measured by the DPPH and ORAC assays existed between regular and decaffeinated coffees. However, there was significant difference of antioxidant capacity in the ABTS assay among all of the coffee brands tested.

Phenolic phytochemicals such as hydroxycinnamic acids and flavonoids are known to be good antioxidants due to their aromatic benzene ring with at least 1 hydroxyl group. A positive linearity was usually obtained between total phenolics and antioxidant capacity (13,26,27). In this regard, the correlation of the phenolic contents vs. antioxidant capacity was determined by Pearson's correlation. In a result, total phenolics exhibited a good linear relationship with antioxidant capacity measured by the ABTS (r^2= 0.946) and DPPH (r^2=0.979) assays, in opposite to the ORAC assay (r^2=0.393, data not shown). The correlation suggested that higher levels of total phenolics in coffees contribute to higher antioxidant capacities. On the other hand, a good correlation was observed between total flavonoids and ORAC (r^2=0.867) as well as ABTS (r^2= 0.629) assays compared to DPPH assay (r^2=0.540, data not shown).

Among 3 chemical assays for the measurements of antioxidant capacity of coffee, the ABTS and DPPH assays had a good correlation (r^2=0.982), whereas the ORAC assay showed relatively lower correlation coefficients with the ABTS (r^2=0.550) and DPPH assays (r^2=0.422) in coffees tested in this study (data not shown). Coffee showed different antioxidant capacity, which might be ascribed to different assays, different cultivars used, and roasting temperature (20-22). Disagreement of antioxidant capacity measured by different assays used in this study seems to be attributed to the difference of correlation coefficients. Data suggest that multiple experimental approaches for the evaluation of antioxidant capacity are inevitable in the future studies.

Protective effect of coffee phenolics on HepG2 cells against oxidative stress Cytotoxicity of coffee phenolics was evaluated by determining the maximum nontoxic concentrations in HepG2 cells. The sample concentrations resulting in over 90% of cell viability were considered to be noncytotoxic. Coffee phenolics showed no cytotoxicity at concentrations less than or equal to 100 μg/mL (data not shown).

Evaluation of cell viability by MTT reduction assay was based on the formation of a purple formazan by the catalytic activity of mitochondrial dehydrogenases in intact cells. The oxidative stress caused by 500 μM of H_2O_2 decreased to approximately 56.6% viability of HepG2 cells compared with the control (100%). Pretreatment of HepG2 cells with coffee phenolics followed by H_2O_2-induced oxidative stress resulted in significantly increased cell viabilities except for the dose of 1 μg/mL of decaffeinated coffee brand D, as compared to non-pretreated positive control. The highest viability (91.3%) of HepG2 cells under oxidative stress was observed at the level of 100 μg/mL of phenolic extract from regular coffee brand B. Pretreatment of cells with phenolics from all brands of regular and decaffeinated coffees increased cell viability in a concentration-dependent manner (Fig. 1A). In accordance, it was also previously reported that regular and decaffeinated coffees protected lymphocytes and macrophages from an oxidative stress in a dose-dependent manner (28).

Cells are vulnerable to oxidative stress partly due to the polyunsaturated fatty acids in the cell membrane. In the case of lipid peroxidation in the plasma membrane, cellular integrity ruptures resulting in the release of cytoplasmic LDH into the medium. Therefore, a quantitative analysis of LDH release in a cell culture medium provides the relative cell viability. As a positive control, HepG2 cells under 500 μM of H_2O_2 and without coffee phenolics had an increase in the percent of LDH in culture medium at 139.3% (Fig. 1B). Pretreatment of the cells with coffee phenolics significantly decreased the amount of LDH release in a dose-dependent manner (Fig. 1B), suggesting coffee phenolics, through their antioxidant capacities, attributed to membrane integrity of HepG2. At concentrations of 10 and 100 μg/mL of coffee phenolics, the release of LDH was significantly reduced compared to that of a positive control. At 1 μg/mL of coffee phenolics, however, no significant reduction of LDH release from HepG2 cells was found compared to a positive control. These results suggest that pretreatment of HepG2 cells with coffee phenolics could attenuate oxidative insult caused by H_2O_2, which may protect mitochondrial function and plasma membrane integrity.

Effect of coffee phenolics on intercellular oxidative stress in HepG2 cells Levels of intracellular oxidative stress caused by ROS were determined with a fluorescent probe, DCFH-DA. DCFH-DA is hydrolyzed by an intracellular esterase to non-fluorescent 2',7'-dichlorofluorescin, which is oxidized to fluorescent 2',7'-dichlorofluorescein under oxidative stress. Exposure of HepG2 cells to H_2O_2 insult resulted in 122.9% increase in oxidative stress level compared to the negative control (100%) without H_2O_2 treatment (Fig. 2). Intracellular oxidative stress was significantly reduced by pretreatments of cells with 1, 10,

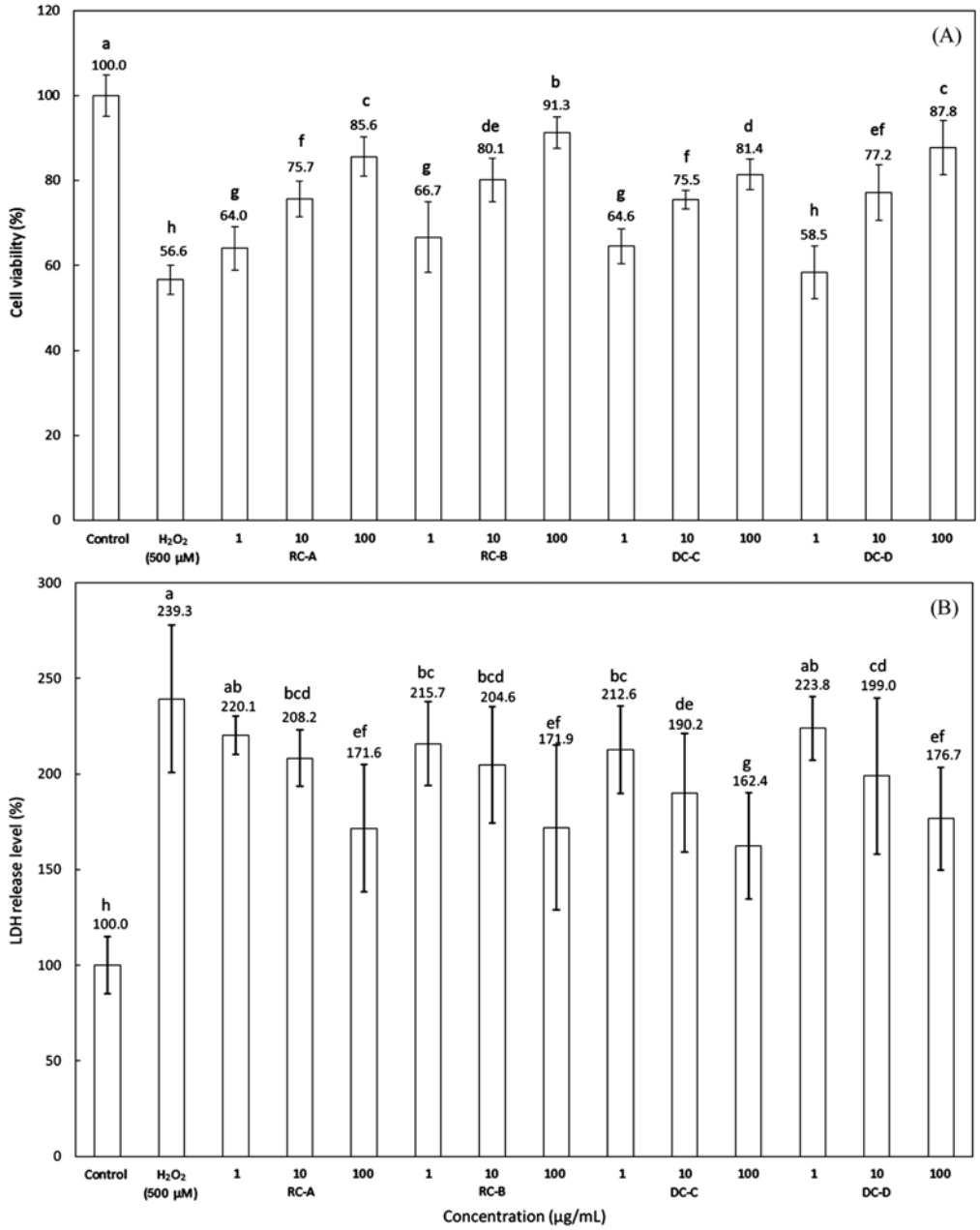

Fig. 1. Protective effects of coffee phenolics on HepG2 cells against oxidative stress caused by H_2O_2 (500 μM) in MTT reduction assay (A) and lactate dehydrogenase (LDH) release assay (B). RC and DC stand for regular (brand A and B) and decaffeinated coffees (brand C and D), respectively. Different letters on bars mean significant differences by Duncan's multiple range test ($p<0.05$, $n=3$).

and 100 μg/mL of phenolic extracts from all brands of regular and decaffeinated coffees except for 1 μg/mL phenolic extract of decaffeinated coffee brand D (Fig. 2). In accordance with aforementioned MTT reduction and LDH release assays (Fig. 1), coffee phenolics diminished intracellular oxidative stress level in a dose-dependent manner. Consequently, the results strongly demonstrated that oxidative stress caused by ROS such as H_2O_2 was overcome by phenolics from regular and decaffeinated coffees.

Effect of coffee phenolics on apoptosis of HepG2 cells against oxidative stress Influence of coffee phenolics on the progress of apoptosis under oxidative stress was evaluated by using flow cytometry. Propidium iodide (PI), which cannot penetrate cellular membranes in living cells, was used a staining marker for dead (necrotic) cells. Apoptotic cells can be detected with a specific binding of FITC-labeled annexin V to a phospholipid, phosphotidylserine (PS), which is moved from the inner leaflet of the cellular membrane to the outer leaflet under apoptosis.

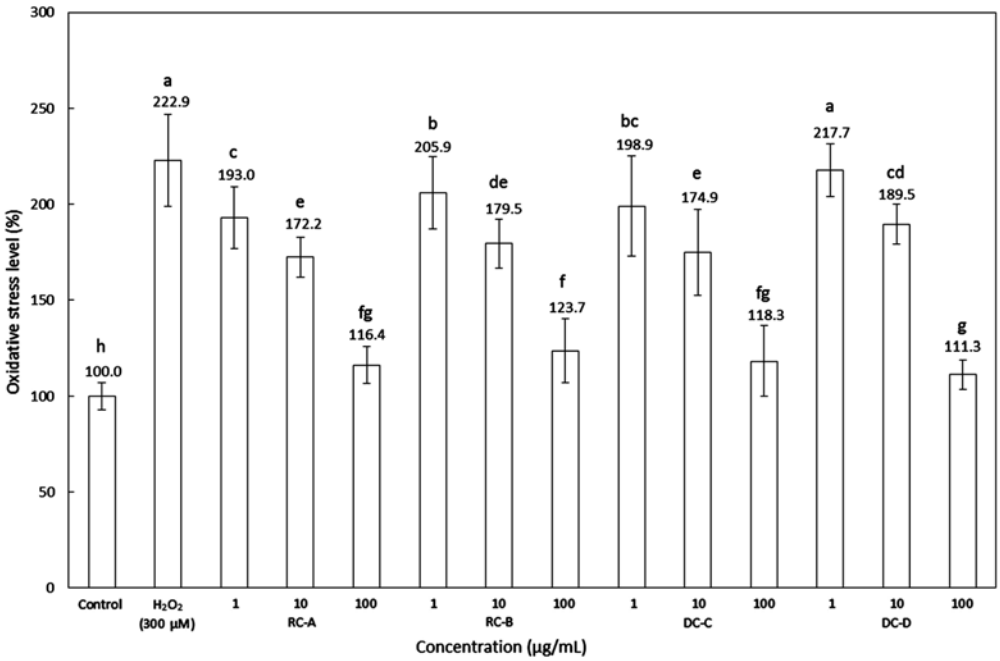

Fig. 2. Effect of coffee phenolics on intracellular oxidative stress on HepG2 cells. RC and DC stand for regular (brand A and B) and decaffeinated coffees (brand C and D), respectively. Different letters on bars mean significant differences by Duncan's multiple range test ($p<0.05$, $n=3$).

Following data acquisition and analysis, annexin V positive cells are in the upper-right and lower-right quadrants, but PI positive cells are in the upper-right and upper-left quadrants (Fig. 3). In a quadrant, dead (necrotic) cells, late apoptotic dead cells, viable cells, and early apoptotic cells are plotted in Q1 (upper left), Q2 (upper right), Q3 (lower left), and Q4 (lower right) quadrant, respectively (Fig. 3). The treatment of H_2O_2, which was previously reported to induce apoptosis (29), to HepG2 cells resulted in the decreased population (45.3%, Fig. 3B) of double negative live cells (annexin V- and PI- (Q3)) compared with control (74.5%, Fig. 3A). In addition, the population of apoptotic cells under oxidative stress was increased to 44.3% (Q3 and Q4, Fig. 3B) compared with control (21.5%, Q3 and Q4, Fig. 3A). In this condition, pretreatment of cells with phenolic extracts from regular coffee brand A resulted in an increase in viable cells (Fig. 3C, 3D) compared to non-pretreated negative control (Fig. 3B). The cell population in apoptosis also decreased from 44.3 to 36.3% at 10 μg/mL of coffee phenolics and 26.5% at 100 μg/mL of coffee phenolics (Fig. 3). These suggest that coffee phenolics prevented apoptosis under oxidative stress.

Figure 4 shows effects of phenolic extracts of 4 brands of regular and decaffeinated coffees on cell viability (% of control) determined by flow cytometry. Compared with control (100%), oxidative stress decreased the cell viability to 55.4%, which means approximately 46.6% of viable cells were in necrosis and/or apoptosis (Fig. 4). Coffee phenolics enhanced viable cell subpopulation in a dose-dependent manner (Fig. 4). At concentration of 10 μg/mL of coffee phenolics, cell viability ranged from 54.2 (brand B) to 67.4% (brand C), where brands A, C, and D exhibited no significant difference from control, but brand B showed significant difference. Cell viability at 100 μg/mL of coffee phenolics ranged from 70.2% (brand D) to 91.2% (brand A), where no significant difference from control was found. Although there was no significant difference among all the groups tested, the overall results suggest that coffee phenolics protect HepG2 cells from necrosis and/or apoptosis and, thus, enhance cell viability under oxidative stress.

Quantification of CQA and its derivatives by HPLC analysis CQA and its derivatives are major components in coffee (6,20,30). Due to catechol moiety on the aromatic ring, CQA and its derivatives effectively scavenge free radicals, and thus account for antioxidant capacity and anti-apoptosis (31-34). Therefore, CQA and its derivatives in regular and decaffeinated coffees tested in this study were quantitatively analyzed by using a reversed-phase HPLC system. The comparison of retention times, UV-visible spectra, and spike-inputs of their standard compounds resulted in the tentative identification of 3-CQA, 5-CQA, 4-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (Table 3). The contents of CQA and its derivatives in 4 brands of coffee in this study decreased as follows: brand B, regular (9.22 mg/g) > brand D, decaffeinated (8.03 mg/g) > brand

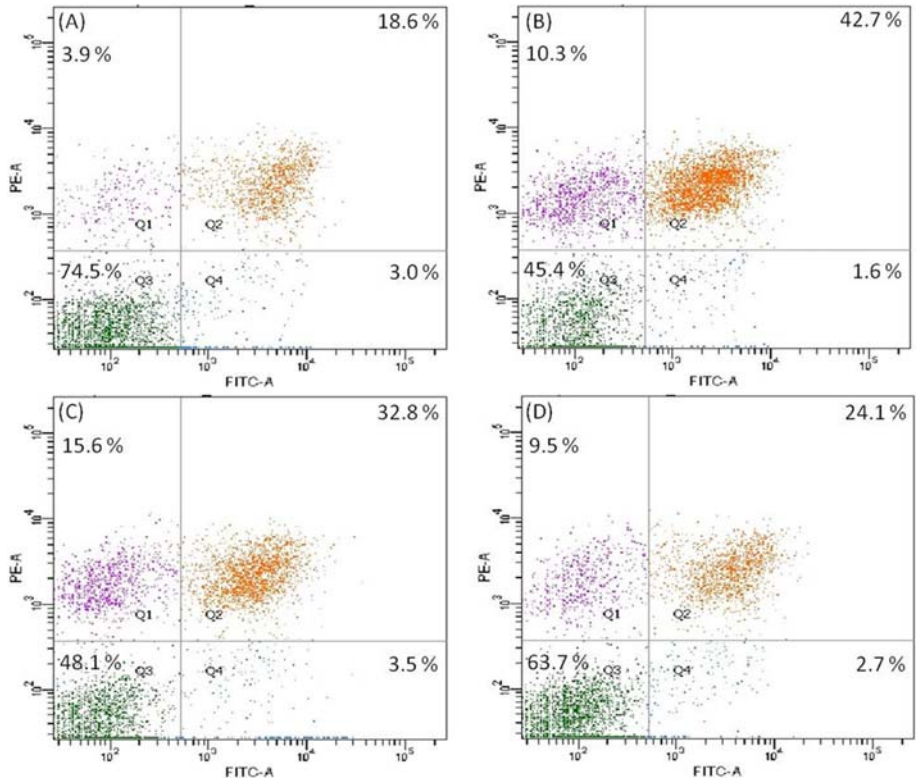

Fig. 3. Effect of coffee phenolics (brand A, regular) on HepG2 cell subpopulations in flow cytometry analysis with FITC-labeled annexin V and propidium iodide. (A) control, (B) 500 μM of H_2O_2, (C) 10 μg/mL of coffee phenolics and 500 μM of H_2O_2, (D) 100 μg/mL of coffee phenolics and 500 μM of H_2O_2

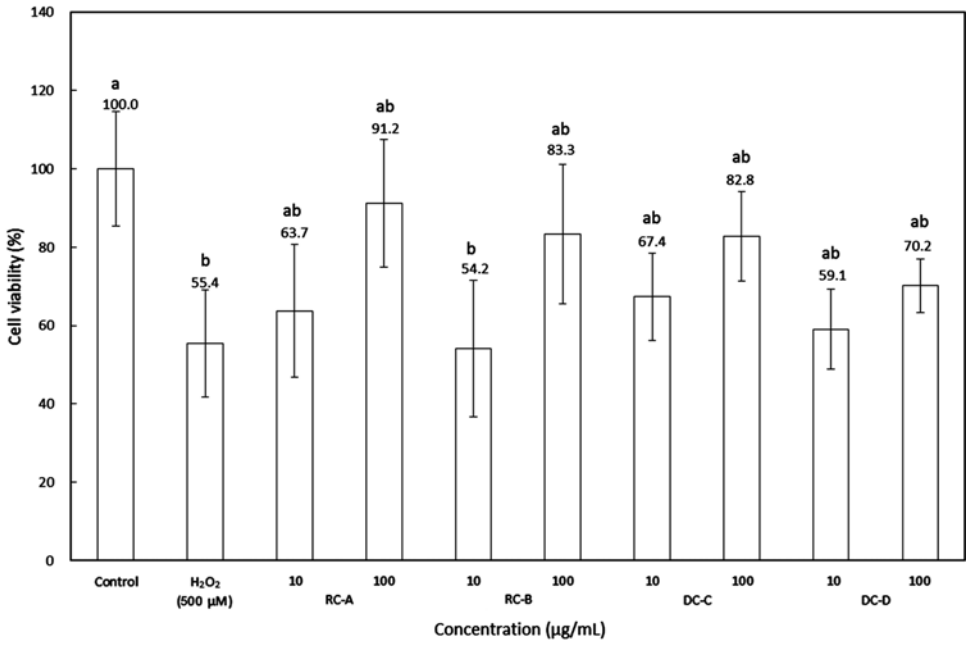

Fig. 4. Anti-apoptotic effect of coffee phenolics on HepG2 cells against oxidative stress caused by H_2O_2 (500 μM) by using flow cytometry and annexin V staining. RC and DC, regular (brand A and B) and decaffeinated coffees (brand C and D), respectively. Different letters on bars mean significant differences by Duncan's multiple range test ($p<0.05$, $n=3$).

A, regular (5.39 mg/g) > brand C, decaffeinated (5.29 mg/g). Among CQA and its derivatives identified, 5-CQA as a major CQA formed 36.9% (brand C, decaffeinated) to 42.8% (brand B, regular). 5-CQA was found to be a predominant

Table 3. Concentration (mg/g) of caffeoylquinic acid (CQA) and its derivatives in regular and decaffeinated coffees commercially available in Korea

Coffee	3-CQA	5-CQA	4-CQA	3,4-diCQA	3,5-diCQA	4,5-diCQA
Regular						
Brand A	1.22±0.06$^{b1)}$	2.09±0.08^{b}	1.47±0.15^{b}	0.28±0.02^{b}	0.13±0.02^{a}	0.20±0.02^{b}
Brand B	1.96±0.27^{a}	3.96±0.49^{a}	2.44±0.40^{a}	0.34±0.03^{a}	0.22±0.02^{a}	0.30±0.05^{a}
Decaffeinated						
Brand C	1.19±0.24^{b}	1.95±0.43^{b}	1.52±0.34^{b}	0.29±0.01^{ab}	0.14±0.09^{a}	0.20±0.04^{b}
Brand D	1.87±0.01^{a}	3.44±0.23^{a}	1.92±0.02^{b}	0.33±0.00^{a}	0.19±0.01^{a}	0.28±0.03^{ab}

[1)]Data are presented as mean±SD (n=3); Different superscripts in the same column show the significant difference by Duncan's multiple range test (p<0.05).

compound among CQA derivatives in accordance with this study (35). A previous finding with no significant difference in chlorogenic acid content between regular and decaffeinated coffees was also in agreement with this study (28).

The total contents of CQA derivatives ranged from approximately 6.0 to 50.17 mg/g (20). The sum of 6 CQA derivatives ranged from 5.29 mg/g (brand C, decaffeinated) to 9.22 mg/g (brand B, regular) in this study. The contents of CQA derivatives were affected by some factors like coffee varieties, roasting degrees, blending, and decaffeination (20,22,35). It was reported that the levels of CQA and its derivatives in roasted coffee were 3-9% lower in decaffeinated coffee compared to regular coffee (35). Regular coffee, however, had no significant difference in 5-CQA content from decaffeinated coffee in this study (28).

In conclusion, coffee, regular or decaffeinated, had considerable amounts of phenolics, accounting for a high level of antioxidant capacity as determined by multiple chemical scavenging assays. Both regular and decaffeinated coffees showed a significantly *in vitro* protective effect in human hepatic epithelial cell line, HepG2 cells, against oxidative stress in a dose-dependent manner. Apoptosis evaluated by using flow cytometry and annexin V staining was effectively inhibited by coffee phenolics, which resulted in the enhanced viability of HepG2 cells. Caffeoylquinic acid (CQA) and its derivatives identified by HPLC included 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA, where 5-CQA as a major component comprised approximately 36.9 to 42.8%. Taken together, the present study supports that coffee leads to protection of cells *in vitro* against overwhelming oxidative stress due to its richness in phenolics, especially CQA and its derivatives. Coffee, regular or decaffeinated, may serve as a good source of health-beneficial phytochemicals in diet.

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