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Inhibitory Effect of the Phenolic Compounds from Apples Against Oxidative Damage and Inflammation

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Abstract - ROS have been associated with pathogenic processes including carcinogenesis through direct effect on DNA and play an important role in the pathogenesis of inflammation. Because of many types of phenolic acid derivatives and flavonoids, apples have been one of the human diet since ancient times and are one of the most commonly consumed fruits in worldwide. In this study, catechin, chlorogenic acid and phlorizin dihydrate were purified and identified by HPLC and GC/MS. The contents of catechin, chlorogenic acid and phlorizin dihydrate were 1.01 mg, 7.01 mg and 3.67 mg/ kg wet weight, respectively. Catechin and phlorizin dihydrate were found to significantly inhibit oxidative DNA damage, while chlorogenic did not affect. Also, catechin inhibits NO and PGE_2 production via suppressing iNOS and COX-2 expression. However, chlorogenic acid and phlorizin dihydrate did not affect. Our results show that catechin may be the most active phenolic compound in anti-oxidative damage and anti-inflammatory effect.

Key words - Apple, Phenolic compounds, Reactive oxygen species (ROS), Anti-oxidative damage, Anti-inflammatory effect

Introduction

Aerobic organisms constantly produce low levels of reactive oxygen species (ROS) (Halliwell, 1994). In some cases, ROS are produced specifically to serve essential biological functions, whereas in other cases, they represent by-products of metabolic processes. All ROS have the potential to cause damage to cellular components including DNA. Some oxidative DNA lesions are promutagenic and oxidative damage is proposed to play a role in the development of certain cancers (Ames, 1989). During normal cellular activities, various processes inside the cell produce ROS such as hydrogen peroxide (H_2O_2), superoxide ion (O^{2-}) and hydroxyl radical (OH^-). The most important target for ROS in the carcinogenesis process is DNA (Lopaczynski and Zeisel, 2001). When present in a high enough concentration, these compounds can damage cellular proteins and DNA, or form DNA adducts that may promote carcinogenic activity (Seifried *et al*., 2007). Also, Excessive ROS (e.g. hydrogen peroxide) can lead to DNA oxidation, causing cell damage to all cellular constituents. Irreparable DNA damage is involved in carcinogenesis, aging and other degenerative diseases (Cozzi *et al*., 1997). Therefore, the protection of oxidative DNA damage induced by ROS is very important for cancer prevention. Also, ROS play an important role in the pathogenesis of inflammation (Amin *et al*., 2004). Innate immunity is an ancient form of host defense that is activated rapidly to enable, through a multiplicity of effector mechanisms, defense against bacterial or viral infections and stresses; however, excessive innate immunity responses may cause chronic inflammation (Brown *et al*., 2007). Chronic inflammation represents a major pathological basis for tumor development. Although inflammation acts as host defense mechanism against infection or injury and is primarily a self limiting process, inadequate resolution of inflammatory responses lead to various chronic disorders associated with cancers. Macrophages are key players in the immune response to foreign invaders such as infectious microorganisms.

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Macrophages are activated by IFN-γ, pro-inflammatory cytokines (Berenbaum, 2000), and bacterial lipopolysaccharide (LPS) (Zhang and Ghosh, 2000). Activated macrophages play an important role in inflammatory diseases via production of nitric oxide (NO) (Fujiwara and Kobayashi, 2005). Its excess production is involved in many diseases including rheumatoid arthritis, atherosclerosis, asthma and pulmonary fibrosis (Bharat Reddy and Reddanna, 2009). Important inflammatory mediator linking chronic inflammation and cancer is NO, which is produced endogenously during arginine metabolism by different isoforms NOS (Moncada *et al*., 1991). During inflammation, induced expression of iNOS in macrophages and epithelial cells leads to production of NO. The expression of iNOS and the level of NO have been shown to be elevated in various precancerous lesions and carcinomas. Thus, pharmacological inhibition of NO and iNOS expression offers promising targets for chemoprevention in inflammation-associated carcinogenesis.

The high participation of fruits and vegetables in the human diet, because of their ability to neutralize active oxygen species, hazardous for health, is of utmost importance. Plant tissue antioxidant capacity is closely associated with activity of "free radical scavenging enzymes" (superoxide dismutase, catalase, peroxidase) and with the contents of antioxidant substances, mainly phenolic compounds, carotenoids, tocopherol and ascorbic acid (Bartosz, 1997). Recently, antioxidant activity has been determined in many species of fruits, vegetables, herbs, cereals, sprouts and seeds. Especial attention is paid to fruits, as rich sources of phenolic compounds. Among others, the antioxidant properties of apple polyphenols have been extensively examined. The apple phenolics, localized mainly in cortex and in skin are compounds with strong antioxidant activity (Leja *et al*., 2003).

Apples are one of the main sources of flavonoids in the Western diet, together with tea, wine, onions, and chocolate (Hertog *et al*., 1993; Art *et al*., 2001). Apples contain as much as 2 g of phenols per kilogram wet weight, or about 400 mg total phenols per apple (Scalbert and Williamson, 2000). The main classes of polyphenols in apples are flavonoids, such as flavonols (quercetin, as glycosides), flavanols ([－]-epicatechin, [+]-catechin, and their oligomers, procyanidins), anthocyanins, and certain dihydrochalcones only found in apples (phloridzin and phloretin), as well as other phenolic compounds, such as chlorogenic acid. These phenolic compounds are responsible for most of the antioxidant activities in apples (Wu *et al*., 2007).

In this study, we isolated three phenolic compounds such as catechin, chlorogenic acid and phlorizin dihydrate from apples. And we investigated whether these isolated phenolic compounds have anti-oxidative damage and anti-inflammatory properties in the non-cellular system and cellular system.

Materials and Methods

Chemical regents

All chemicals for the purification and identification of phenolic compounds from apples were obtained from Sigma Chemicals Co. (St. Louis, USA). φX-174 RF I plasmid was purchased from New England BioLabs (County Road Ipswich, MA). LPS (Escherichia coli 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies recognizing iNOS and COX-2 were purchased from Santa Cruze Biothechnology (Santa Cruz, CA, USA). And phospho-H2AX antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary antibody, goat anti-rabbit IgG-HRP conjugated, for lunasin peptide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa 488, the secondary antibody for immuno-staining was purchased from Invitrogen (Carlsbad, CA, USA). Prostaglandin E2 ELISA monoclonal Kit and Nuclear Extraction Kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). Dulbeco's modified Eagle's medium (DMEM), penicillin and fetal bovine serum (FBS) were purchased from Gibco Inc. (NY, USA). All electrophoresis chemicals were purchased from Bio-Rad Labs (Hercules, CA, USA).

Isolation and identification of catechin, chlorogenic acid and phlorizin dihydrate from apples

Apples (cv. Fugi) were kindly obtained from Cheongsong Agriculture Technology & Extension, Andong, Korea. Two kilogram of apples was extracted with 3L of 80% methanol with shaking for 24 h. After 24 h, extract with 80% methanol

was filtered, concentrated to approximately 600 ml volume using by a vacuum evaporator, and fractioned with n-hexane, chloroform and ethyl acetate in consecutive order in a separating funnel. Ethyl acetate fraction was collected, evaporated by a vacuum evaporator, and kept at -80°C for the purification of catechin, chlorogenic acid and phlorizin dihydrate.

Twenty micro liter of ethyl acetate fraction (4 mg/ml methanol) was injected into HPLC equipped with C18 column (DELTA PAK, 15 μm, 300 A, 300 × 7.8 mm) equilibrated at ambient temperature and stabilized with the mobile phase (ratio of 1.6 to 8.4=methanol:water) at a flow rate of 1 ml/min for 40 min at the UV detector set at 280 nm. Catechin, chlorogenic acid and phlorizin dihydrate were identified by being compared with the retention time of the standards, respectively. And the recoveries were calculated by being compared to the peak areas of the standards subjected to the same treatments. Purified catechin, chlorogenic acid and phlorizin dihydrate was collected with a fraction collector and then stocked in -20°C for the identification. Also we measured the amount of catechin, chlorogenic acid and phlorizin dihydrate from apples through comparing the peak area of standards. The quantitative analysis was carried out three times.

For identification, purified catechin, chlorogenic acid and phlorizin dihydrate were analyzed by GC−MSD, equipped with a Supelcowax 10 fused silica capillary (30 m length × 0.25 mm i.d. supelco, USA). The carrier gas used was helium, at a constant flow rate of 1.0 ml/min. Two microliter of purified catechin, chlorogenic acid and phlorizin dihydrate was injected into the column using 10:1 of the split ratio injection mode. The oven temperature was initially held at 100°C for 3 min, then raised to 300°C for 5 min, and finally held at 300°C for 48 min. The temperatures of injector and detector were 200°C and 240°C, respectively. The mass detector was operated in electron impact mode with an ionization energy of 70 eV, a scanning range of 33−550 a.m.u. and a scan rate of 1.4 scans/s. Purified catechin, chlorogenic acid and phlorizin dihydrate were positively identified by comparing the mass spectra and RIs in the Wiley 275 Imass spectral database (Hewlett-Packard, 1995).

φX-174 RF I plasmid DNA damage assay

Conversion of the supercoiled form of plasmid DNA to the open-circular and further linear forms has been used as an index of DNA damage (Jung and Surh, 2001). Reaction mixtures (25 μl) contained 5 μl of φX-174 RF I plasmid DNA, 10 μl of varying concentrations of purified catechin, chlorogenic acid and phlorizin dihydrate, 5 μl of 1 mM $FeSO_4$ and 5 μl of 1 mM H_2O_2 and were incubated at 37°C for 30 min. After 30 min, 5 μl of a solution containing 50% glycerol (v/v), 40 mM EDTA and 0.05% bromophenol blue was added to stop the reaction and the reaction mixtures were electrophoresed on 1% agarose gel, and the DNA in the gel was visualized and photographed under ultraviolet light after ethidium bromide staining.

Western blot of phosphorylated H2A.X in hydroxyl radical-treated NIH 3T3 cells

NIH 3T3 cells (2×10^6) were cultured in 6-well plates with or without a cover glass for 24 h at 37°C. After 24 h, purified catechin was treated and then incubated for 1 h at 37°C. After 1 h, total concentration of 1 mM $FeSO_4$ and H_2O_2 was treated and then the cells were incubated for 24 h at 37°C. For Western blot analysis, after 24 h, each cells were lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM DTT) containing 1 % Triton X-100. Insoluble debris was removed by centrifugation at 15,000 g for 15 min three times. The protein extracted from the cells was separated on 15 % Tris-HCl ready gel (Bio-rad, Hercules, CA) following the manufacturer's instruction. Gels were transblotted on to PVDF membranes. The membranes were blocked for non-specific binding for 90 min in block buffer (5 % non-fat milk and 0.1 % Tween 20 in 1×Tris-buffered saline[TBS]) and then washed with 1×TBS solution (0.1% Tween 20 in 1×TBS). After washing with 1×TBS solution, the membrane was incubated with phospho-H2A.X primary antibody at 1:500 dilutions in antibody dilution buffer (5% BSA, 1×TBS, 0.1% Tween 20) with gentle shaking at 4°C for 16 h and then washed with 1×TBS solution. After washing, the membranes were incubated with Phototope-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody as the secondary

antibody at 1:500 dilutions in antibody dilution buffer (5% non-fat milk and 0.1% Tween 20 in 1×TBS) for 1 h and then washed again. After washing, the membranes were treated with the detection agent (Amersham Biosciences) and immediately developed in Polaroid film. For immuno-staining analysis, the cells were fixed with 2% formaldehyde for 1 h and then washed with 1×PBS for 5 min three times. PBS with 10% fetal bovine serum (PBS/FBS) was then added to block non-specific binding for an hour. After washing, anti phospho-H2A.X polyclonal anti-body was diluted in 0.1% saponin/PBS/FBS solution at 1:250 dilutions for 18 h at 4°C with gently shaking, and then washed with 1×PBS for 5 min three times. Subsequent antibody, Alexa-Flour 488 goat anti-rabbit IgG, and DAPI were used against phospho-H2A.X antibody and nuclei, respectively and the cells were incubated in the dark for an hour. Then, the cells were washed with 1×PBS and mounted with antifade mounting medium. Mounted slides were viewed under a fluorescence microscope using a 60×oil immersion objective. The excitation wavelengths for DAPI and phospho-H2A.X were 359 nm and 494 nm, respectively.

Determination of NO and PGE_2 production in LPS-stimulated RAW264.7 cells

Inhibitory effects of purified catechin, chlorogenic acid and phlorizin dihydrate on the production of nitric oxide (NO) in RAW 264.7 cells was evaluated using a method modified from that previously reported (Banskota *et al*., 2003). RAW 264.7 cells (2×10^5 cells/well) in 10% FBS-DMEM without phenol red were seeded in a 6-well plate for 24 h at 37°C. Cells were washed with 1 X PBS, replaced with fresh media, and then treated with the varying concentrations of purified catechin, chlorogenic acid and phlorizin dihydrate for 1 hour. LPS (1 μg/ml) was treated for 24 h at 37°C. After 24 h, 200 μl of the medium were placed in a 96-well plate and an equal amount of Griess reagent (1% sulfanilamide and 0.1% N-1-(naphthyl) ethylenediamine-diHCl in 2.5% H_3PO_4) was added. The plate was incubated for additional 5 min at the room temperature and then the absorbance was measured at 540 nm with a SepectraMax 340 microplate reader. The amount of nitric oxide was calculated using sodium nitrite standard curve. For the measurement of Prostaglandin E_2 (PGE_2) production, 50 μl of the supernatant of cultured medium was collected, and PGE_2 production was determined using prostaglandin E2 ELISA monoclonal Kit with manufacturer's instructions.

Western blot for iNOS and COX-2 in LPS-stimulated RAW264.7 cells

RAW 264.7 cells (2×10^5 cells/well) were seeded in a 6-well plate for 24 h at 37°C. Cells were washed with 1×PBS, replaced with fresh media, and then treated with the varying concentrations of purified catechin, chlorogenic acid and phlorizin dihydrate for 1h. After 1 h, LPS (1 μg/ml) was treated for 24 h at 37°C. Each cell was harvested and lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM DTT) containing 1 % Triton X-100. Insoluble debris was removed by centrifugation at 12,000 g for 15 min three times. Fifty microgram of each protein from the cells was separated on 8% Tris-HCl ready gel (Bio-rad, Hercules, CA) following the manufacturer's instruction. Gels were trans-blotted on to PVDF membranes. The membranes were blocked for non-specific binding for 90 min in block buffer (5% non-fat milk in 1×TBS solution containing 0.1% Tween-20) and then washed with 1×TBS solution (0.1% Tween-20 in 1×TBS). The membranes were incubated with iNOS and COX-2 antibody at 1:1000 dilutions in antibody dilution buffer (3% non-fat milk in 1×TBS containing 0.1% Tween-20) with gentle shaking at 4°C for 18 h and then washed with 1×TBS. After washing, the membranes were incubated with Phototope-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody as the secondary antibody at 1:1000 dilutions in antibody dilution buffer (3% non-fat milk in in 1×TBS solution containing 0.1% Tween-20) for 1 h at the room temperature and then washed again. After washing, the membranes were treated with the detection agent (Amersham Biosciences) and immediately developed in Polaroid film.

Statistical analysis

All results were expressed as the mean ± the standard deviation of triplicate analysis. Statistical comparisons were

performed using the Student's t-test. Differences were considered significant at $p < 0.05$.

Results

Purification and identification of catechin, chlorogenic acid and phlorizin dihydrate from apples

Purification and identification of catechin, chlorogenic acid and phlorizin dihydrate from apples were carried out by HPLC and GC/MS analysis. Fig.1 and 2 show the HPLC chromatogram and GC/MS spectrums of catechin, chlorogenic acid and phlorizin dihydrate from apples. The retention time of the HPLC chromatogram of purified catechin, chlorogenic acid and phlorizin dihydrate from apples was in accord with that of its standards. Also in GC/MS analysis, purified catechin, chlorogenic acid and phlorizin dihydrate from apples were identified as authentic catechin, chlorogenic acid and phlorizin dihydrate in the Wiley 275 Imass spectral database (Hewlett-Packard, 1995) by being in accord with the GC/MS spectrum of catechin, chlorogenic acid and phlorizin dihydrate from apples. Also we measured the amount of catechin, chlorogenic acid and phlorizin dihydrate from apples through comparing the peak area of standards. The total amount of catechin, chlorogenic acid and phlorizin dihydrate from apples was approximately 1.01 ± 0.45, 7.01 ± 0.36 and 3.67 ± 0.42, respectively (Table 1).

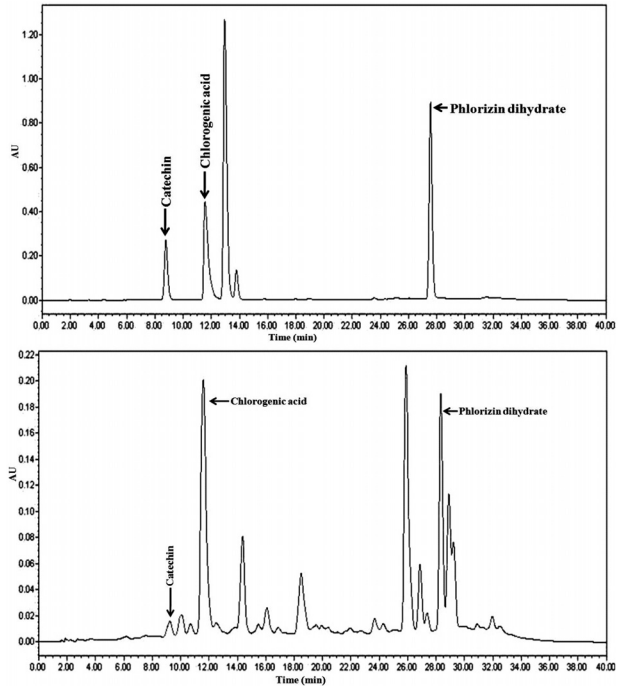

Fig. 1. Purification and identification of catechin, chlorogenic acid and phlorizin dihydrate from apples. HPLC chromatogram of standards (upper panel) and crude extracts from apples (lower panel).

Catechin, chlorogenic acid and phlorizin dihydrate from apples prevent DNA from oxidative damage in the non-cellular system and cellular system

The plasmid DNA cleavage assay using phi X-174 RF I plasmid DNA was used as an initial approach toward

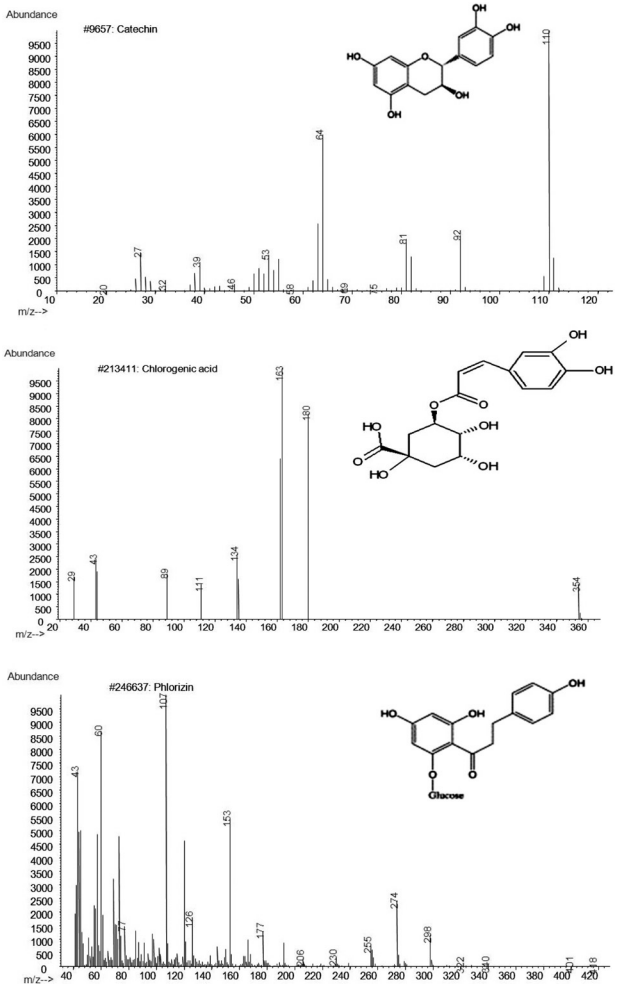

Fig. 2. Chemical structures and GC/MS spectrums of purified catechin (upper panel), chlorogenic acid (middle panel) and phlorizin dihydrate (lower panel). In identification by GC/MS, phenolic compounds were identified with the aid of the Wiley 275 Imass spectral database (Hewlett-Packard, 1995).

Table 1. Contents of catechin, chlorogenic acid and phlorizin dihydrate from apples.

Phenolic compounds	Contents (mg / kg wet weight)
Catechin	1.01 ± 0.45
Chlorogenic acid	7.01 ± 0.36
Phlorizin dihydrate	3.67 ± 0.42

determining whether catechin, chlorogenic acid and phlorizin dihydrate from apples could protect DNA from oxidative damage induced by hydroxyl radical. In this assay, induction of single strand breaks to supercoiled double stranded plasmid DNA leads to formation of open circular DNA, while the formation of a linear form of DNA is indicative of double strand breaks (Li and Trush, 1993). Fig. 3A shows gel electrophoretogram of the protective effect of catechin, chlorogenic acid and phlorizin dihydrate from apples on the cleavage of the plasmid DNA by hydroxyl radical. As observed in Fig. 3A, the plasmid DNA was mainly super-coiled form (SC) in the absence of hydroxyl radical. In the treatment of hydroxyl radical without catechin, chlorogenic acid and phlorizin dihydrate from apples, SC was completely converted into open-circular form (OC). However, in presence of hydroxyl radical, addition of catechin, chlorogenic acid and phlorizin dihydrate from apples inhibited the conversion of SC into OC and linear form (LC). At 250 μg/ml, catechin and phlorizin dihydrate significantly inhibited oxidative DNA damage by 82% and 68%, respectively, while chloro-genic acid did not inhibits oxidative DNA damage. In the dose-dependent assay of catechin with the strongest activity on oxidative damage, catechin inhibited oxidative DNA damage by 46% at 62.5 μg/ml, 61% at 125 μg/ml, 79% at 250 μg/ml, and 91% at 500 μg/ml.

Also, protective effect of catechin against intracellular DNA damage induced by hydroxyl radical was evaluated by the expression level of phospho-H2AX by Western blot and immuno-staining in the cellular system using NIH 3T3 cells. The phosphorylation of H2AX, a sensitive marker for breaks of double stranded DNA (Rogakou *et al*., 1998). In Western blot analysis of phospho-H2AX (Fig. 3B), hydroxyl radical induced hyper-phosphorylation of H2AX compared with the cells without hydroxyl radical and catechin, which means the induction of oxidative DNA damage. However, the treatment

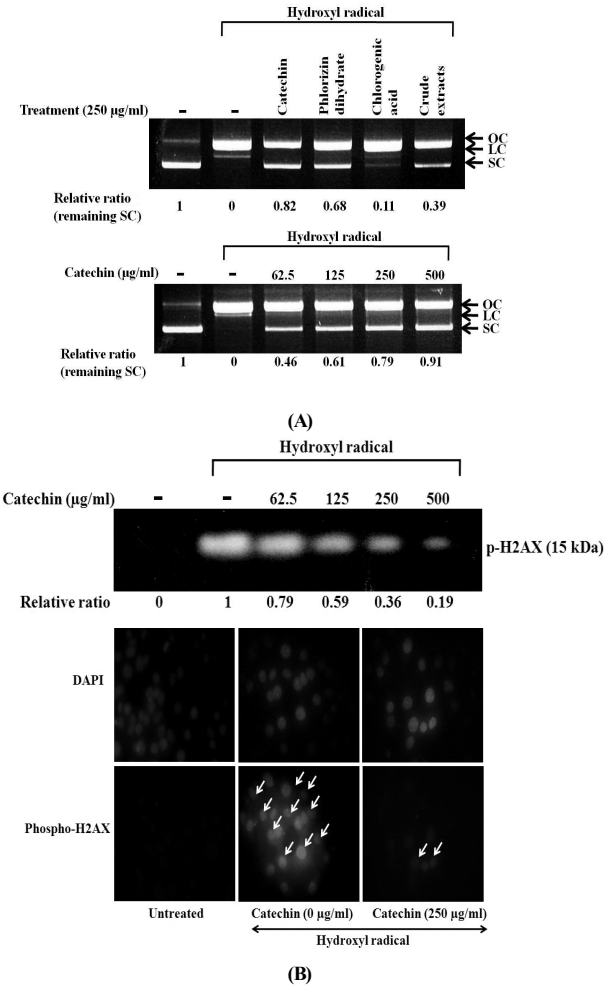

Fig. 3. Anti-oxidative DNA damage of catechin, chlorogenic acid and phlorizin dihydrate from apples in the non-cellular system using phi-X 174 RF I plasmid DNA (A) and in the cellular system using NIH 3T3 cells (B). In Fig. A., SC, OC and LC mean supercoiled form, open-circular form and linear form, respectively. Relative ratio of remaining SC was measured using the software Un-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.). In Fig. B., upper and lower images show Western blot and immuno-staining for the expression level of phospho-H2AX. In SDS-PAGE, 50 μg protein was separated with 15 % Tris-HCl gel. Relative ratio was measured using the software Un-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.).

of catechin dose-dependently inhibits the phosphorylation of H2AX by 21% at 62.5 μg/ml, 41% at 125 μg/ml, 64% at 250 μg/ml, and 81% at 500 μg/ml. This result was confirmed by changes of phospho-H2A.X foci into the nucleus in immuno-staining. From these results, it is thought that catechin inhibits oxidative DNA damage.

Catechin inhibits NO and PGE_2 production via suppressing iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells

iNOS acts on L-arginine to produce NO, which is strongly associated with cytotoxicity and tissue damage and is involved in several processes such as inflammation and immunoregulation (Sung *et al*., 2009). To determine whether catechin, chlorogenic acid and phlorizin dihydrate from apples inhibits the production of NO induced by LPS which plays a central role in the inflammatory response, RAW264.7 cells were pretreated with catechin, chlorogenic acid and phlorizin dihydrate from apples for 1 h and then stimulated with LPS (1 μg/ml). After stimulation for 24 h, the cell medium was harvested, and the production of NO was measured using the Griess assay. RAW264.7 cells unstimulated by LPS secreted basal level of NO, while the stimulation of LPS without catechin, chlorogenic acid and phlorizin dihydrate from apples resulted in an increase in NO production. Catechin significantly does-dependently inhibited the production of LPS-induced NO, while chlorogenic acid and phlorizin dihydrate did not inhibit LPS-induced NO production (Fig. 4A).

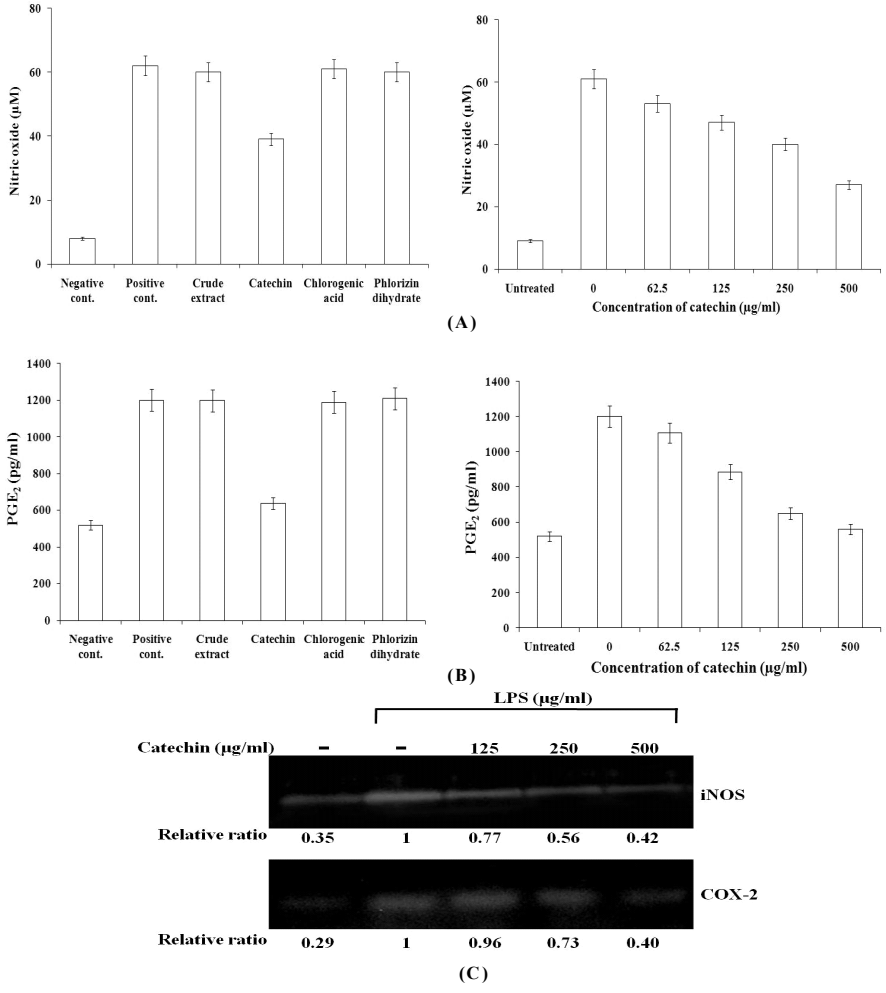

Fig. 4. Anti-inflammatory effect of catechin, chlorogenic acid and phlorizin dihydrate from apples in LPS-stimulated RAW264.7 cells. Cells were pre-treated with catechin, chlorogenic acid and phlorizin dihydrate from apples for 1 h and then 1 μg/ml of LPS was treated for 24 h. (A) The NO concentration in the medium was measured using Griess reagent. (B) The PGE_2 concentration in the medium was measured using Prostaglandin E2 ELISA monoclonal Kit. Each bar of the plot represents the mean ± S.D. from three independent experiments. In western blot analysis (C), equal amount of protein was separated with 8% Tris-HCl gel. Relative ratio was measured using the software Un-SCAN-IT gel Version 5.1 (Silk Scientific, Inc.).

PGE_2 is an inflammatory mediator which is produced from the conversion of arachidonic acid by cyclooxygenase. In a variety of inflammatory cells, including macrophages, COX-2 is induced by cytokines and other activators, such as LPS, resulting in the release of a large amount PGE_2 at inflammatory sites (Yoon *et al*., 2009). Therefore, we evaluated the inhibitory effects of catechin, chlorogenic acid and phlorizin dihydrate from apples against PGE_2 production in LPS-stimulated RAW264.7 macrophages. As shown in Fig. 4B, when stimulated with LPS (1 μg/ml) without catechin, chlorogenic acid and phlorizin dihydrate from applesfor 24 h, RAW264.7 macrophages produced PGE_2 by 1208 pg/ml in the culture medium. However, catechinsignificantly inhibited the production of LPS-induced PGE_2, while chlorogenic acid and phlorizin dihydrate did not affect the production of LPS-induced PGE_2.

To further understand the inhibitory effects of catechin against NO and PGE_2 production, Western blot analysis was performed to determine the expression of iNOS and COX-2 protein at 24 h after LPS stimulation. Catechin attenuated the iNOS and COX-2expressions in LPS-stimulated RAW264.7 cells in a concentration-dependent manner compared with RAW264.7 cells stimulated by LPS without catechin (Fig. 4C). This implies that catechin inhibited NO and PGE_2 production by suppressing iNOS and COX-2 expression in LPS-stimulated RAW264.7 cells.

Discussion

Polyphenols are widely common secondary metabolites of plants, the content of which varies greatly between different species, and cultivars, and with maturity, season, region and yield. Polyphenols are classified according to their structure as phenolic acids derivatives, flavonoids, stilbenes or lignans (Harborne, 1988). They are further sub-divided on the basis of the hydroxylation of phenolic rings, glycosylation, acylation with phenolic acids and the existence of stereoisomers. They are present in many beverages (e.g., red wine and green tea) and foods (e.g., chocolate, grapes, and apples). Several recent studies have reported physiological functionalities of polyphenols (Shoji *et al*., 2004).

Apples have been one of the human diets since ancient times and are one of the most commonly consumed fruits in worldwide. They are eaten both raw and in processed products such as juice, cider, brandy, jam and vinegar. Apples contain many types of phenolic acid derivatives and flavonoids (flavan-3-ols, flavonols, procyanidins, chalcones, and anthocyanins) (Spanos *et al*., 1990; Lister *et al*., 1994). Apple polyphenols have been reported to have various physiological functions including in vivo and clinical anti-allergic activity (Kanda *et al*., 1998; Akiyama *et al*., 2000; Kojima *et al*., 2000), in vivo anti-caries activity (Yanagida *et al*., 2000), and in vitro and in vivo inhibitory activity against some enzymes and receptors (Shoji *et al*, 2000).

In this present study, we isolated and identified catechin, chlorogenic acid and phlorizin dihydrate from apple. Also, the contents of catechin, chlorogenic acid and phlorizin dihydrate in apples were measured. Among catechin, chlorogenic acid and phlorizin dihydrate, the content of chlorogenic acid was highest as 7.01 mg/kg wet weight, while that of catechin was lowest as 1.01 mg/kg wet weight.

Reactive oxygen species (ROS) have been associated with pathogenic processes including carcinogenesis through direct effect on DNA directly and by acting as a tumor promoter (Kong *et al*., 2001). Radicals have been demonstrated to be initiators of the oxidative process (Pietraforte *et al*., 2002) and to be involved in the development of disease (Stohs, 1995). Catalase, superoxide dismutase, glutathione and uric acid are examples of antioxidants produced by organisms under normal conditions as part of defense system against ROS-mediated cellular injury. However, if this defense system is challenged or overwhelmed by excessive generation of ROS, redox imbalance or oxidative stress may occur. This can result in damage to the organism and disease initiation (Halliwell and Gutteridge, 2000). The health promoting effect of antioxidants from plants is thought to arise from their potential effects on the reactive oxygen/nitrogen species. In addition, antioxidants have been widely used in food industry to prolong the shelf life. However, there is widespread agreement that some synthetic antioxidants such as butylhydroxyanisole and butylhydroxytoluene need to be replaced with natural antioxidants due to their potential health risks and toxicity (Li *et al*., 2008).

Also, ROS play an important role in the pathogenesis of

inflammation (Amin *et al*., 2004). The mechanism of inflammation injury is attributed, in part, to release of reactive oxygen species from activated neutrophils and macrophages. In addition, ROS propagate inflammation by stimulating release of mediators, nitric oxide (NO) and prostaglandin E_2 (PGE_2) which stimulate recruitment of additional neutrophils and macrophages. Thus free radicals are important mediators that provoke or sustain inflammatory processes and, consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation (Geronikaki and Gavalas, 2006). Most clinically important medicines are steroidal or non-steroidal anti-inflammatory chemical therapeutics for treatment of inflammation-related diseases. Though these have potent activity, long-term administration is required for treatment of chronic disease. Furthermore, these drugs have various and severe adverse effects. Therefore, naturally occurring agents, with high effectiveness and very few side-effects, are desirable as substitutes for chemical therapeutics (Menichini *et al*., 2009). However, the anti-oxidative damage and anti-inflammatory effect of the phenolic compounds, especially catechin, chlorogenic acid and phlorizin dihydrate, have not yet been studied.

In the evaluation of anti-oxidative damage, catechin and phlorizin dihydrate significantly inhibits oxidative DNA damage, while chlorogenic acid did not affect the inhibition of oxidative DNA damage induced by hydroxyl radical. In anti-inflammatory effect, catechin significantly attenuated NO and PGE_2 production via suppressing iNOS and COX-2 protein expression, while, chlorogenic acid and phlorizin did not inhibit NO and PGE_2 production. Our results show that catechin may be the most active phenolic compound in anti-oxidative damage and anti-inflammatory effect.

Consequently, it was observed that apples have a potential fruit for anti-oxidative damage and anti-inflammation.

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