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The Isolation and Antioxidative Effects of Vitexin from *Acer palmatum*

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Free radicals and reactive oxygen species (ROS) caused by UV exposure or other environmental factors are critical players in cellular damage and aging. In order to develop a new anti-photoaging agent, this work focused on the antioxidant effects of the extract of tinged autumnal leaves of *Acer palmatum*. One compound was isolated from an ethyl acetate soluble fraction of the *A. palmatum* extract using silica gel column chromatography. The chemical structure was identified as apigenin-8-C-beta-D-glucopyranoside, more commonly known as vitexin, by spectral analysis including LC-MS, FT-IR, UV, ^{1}H-, and ^{13}C-NMR. The biological activities of vitexin were investigated for the potential application of its anti-aging effects in the cosmetic field. Vitexin inhibited superoxide radicals by about 70% at a concentration of 100 μg/mL and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by about 60% at a concentration of 100 μg/mL. Intracellular ROS scavenging activity was indicated by increases in dichlorofluorescein (DCF) fluorescence upon exposure to UVB 20 mJ/cm^2 in cultured human dermal fibroblasts (HDFs) after the treatment of vitexin. The results show that oxidation of 5-(6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) is inhibited by vitexin effectively and that vitexin has a potent free radical scavenging activity in UVB-irradiated HDFs. In ROS imaging using a confocal microscope we visualized DCF fluorescence in HDFs directly. In conclusion, our findings suggest that vitexin can be effectively used for the prevention of UV-induced adverse skin reactions such as free radical production and skin cell damage.

Key words: *Acer palmatum*, Vitexin, Antioxidant, Free radical, Photoprotection

INTRODUCTION

Most symptoms of cutaneous aging and skin cancers are the result of exposure to solar radiation. Evidence of free radical formation in UV-radiated skin (Norins, 1962) provided the basis for the implication of reactive oxygen species (ROS) in UV-mediated cutaneous damage including skin cancer, autoimmune disease, phototoxicity, photosensitivity and skin aging.

A number of antioxidants that possess oxygen radical scavenging properties have been tested as potentially beneficial photoprotective agents from these extrinsic factors (Mathews-Roth, 1983; Khettab *et al.*, 1988).

Intrinsic oxidation-protecting enzyme systems, including superoxide dismutase (SOD), catalase, glutathione peroxidase, and food-derived substances such as tocopherols, flavonoids, ascorbic acid and carotenes are known to diminish the undesired effects caused by oxidation processes in organisms (Shibamoto, 1994).

Recently, some antioxidants have been receiving much attention from researchers and the press because of their presence in fruits and vegetables which are regularly consumed by the population. In particular, flavonoids and related compounds, which are widely distributed in the plant kingdom, show remarkable promise for a wide range of pharmacological uses, including anti-allergic, anti-inflammatory, anti-viral, anti-tumor and anti-diabetic treatments (Middleton *et al.*, 2000). *In vivo* and *in vitro* flavonoids are powerful scavengers of ROS, inhibitors of lipid peroxidation, metal ion chelators, inhibitors haem protein/peroxide damage, and inhibitors of lipoxygenase and cyclooxygenase. In addition, the various beneficial properties of flavonoids come from ROS scavenging

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activity (Nijveldt *et al.*, 2001).

Most flavonoids, except catechins, exist in nature as glycosides. Some glycosylflavonoids directly bind the anomeric carbon of a sugar to the nucleus carbon of flavonoids. In general, the antioxidant activity of flavonoids depends on the molecular structure, the degree of hydroxylation and glycosylation, and the positions of hydroxyl groups and glycosyl groups (Middleton *et al.*, 2000; Harborne, 1986; Bors *et al.*, 2002).

Processes involved in plant growth vary by changes in the season. During autumn, green leaves gradually turn red or yellow because these colors originate from xanthophyll, carotenoids and flavonoids. In terrestrial higher plants, any stress (e.g., low temperatures, drought or nutrient deficiencies) reduces the capacity of photosynthetic carbon metabolism (Dauborn *et al.*, 1998). Increased sensitivity to photo-inhibitory conditions during leaf senescence may also be a consequence of deterioration in a plants normal photo-protective mechanisms. Afitlhile *et al.* and Young *et al.* detected reduced amounts of xanthophyll cycle pigments during senescence, indicating that at least some species may suffer diminished amounts of zeaxanthin-related protection as leaves senescence (Afitlhile *et al.*, 1993; Young *et al.*, 1991). After production of chlorophyll stops, trees can produce another leaf pigment including flavonoids and related compounds that creates the colored seen in red maple and senescent birch leaves (Feild *et al.*, 2001; Riipi *et al.*, 2002).

Secondary metabolites are present in a higher quantity compared to green leaves, which contain abundant primary metabolites such as water, amino acids, carbohydrates and proteins. Therefore, autumnal leaves would be expected to contain increased levels of anti-oxidative secondary metabolites capable of guarding against ROS that are formed during photo-inhibition.

Vitexin, cyanidin galloylglucoside, cyanidin acetylrutinoside and other flavonoids were isolated from *Acer. palmatum* (Aritomi, 1963, 1964). Among these compounds, Prabhakar *et al.* reported potent hypotensive effects of vitexin by its ganglion blocking properties. Additionally, anti-inflammatory effects of vitexin were indicated by its anti-histamic, anti-bradykinin and anti-serotonin properties (Prabhakar *et al.*, 1981). Gaitan *et al.* reported anti-thyroid, anti-arteriosclerotic, anti-hypertensive, and anti-hepatotoxic effects of vitexin from millet and hawthorn (Gaitan *et al.*, 1995).

In this study, we isolated vitexin from tinged autumnal leaves of *A. palmatum* and investigated its anti-oxidative properties.

MATERIALS AND METHODS

Materials and instruments

Epigallocatechin-3-gallate (EGCG) and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO, USA). The fluorogenic probes, 5-(6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) and pluronic F-127 were purchased from Molecular probes (Eugene, OR, USA). Melting points were determined with Mel-temp II (laboratory devices, Holliston, MA, USA). IR spectra were obtained with Jasco FT-IR 5300 (Jasco co., Tokyo, Japan) and UV-VIS spectra were obtained using the Varian Cary IE spectrophotometer (Varian, Inc., Palo Alto, CA, USA). ^{1}H-(300 MHz) and ^{13}C-(75 MHz) NMR spectra were obtained using the Bruker 300 MHz NMR spectrometer (Bruker Instruments, Inc., Billerica, MA, USA). The LC-MS spectrum was obtained using the Waters alliance 2695 LC-MS (Waters, USA).

Extraction and isolation

The tinged autumnal leaves of the maple tree (*Acer palmatum* THUNBERG., *Aceraceae*) were selected between October and November in the central region of South Korea. Extracts of 100 g of leaves were made with 5 L of ethanol for 10 days at room temperature and the extract was evaporated *in vacuo* at 40 °C. The ethanol extract (13.4 g) was suspended in water and subsequently partitioned with hexane (1.4 g), chloroform (3.2 g), EtOAc (1.8 g), and BuOH (2.7 g), consecutively. Anti-oxidative activity of each of the four layers was examined by the nitroblue tetrazolium (NBT) test to select the layer which contained the most active substances (Table I). The EtOAc extract was chromatographed on a silica gel column (3.0 × 35 cm) using stepwise gradient elution with the solvents chloroform-MeOH (9:1 → 1:1 v/v) which divided the fraction into six subfractions (Fr. I VI, Table II). Compound 1 (110 mg) was obtained by crystallization in MeOH from subfraction V.

Apigenin-8-C-beta-D-glucopyranoside, vitexin (1)

Amorphous yellowish powder; mp; 250-252 °C; LC-MS *m/z*: 433 $[M+1]^+$; IR (KBr) 3383, 1655 (α, β-unsaturated

Table I. Antioxidative activity of the ethanol extract and its solvent soluble fraction from *Acer palmatum*

Samples	Antioxidative activity
	Inhibition ratio (%)*
Ethanol	81.2
Hexane	28.6
Chloroform	53.7
EtOAc	84.5
BuOH	62.4

* Antioxidative activity is the percent of inhibition of superoxide radicals at the test concentration of 100 μg/mL. Values of antioxidative activity are expressed as the mean of three experiments.

Table II. Antioxidative activity of the subfractions from EtOAc extract

Samples	Antioxidative activity
	Inhibition ratio (%)*
Fraction I	32.7
Fraction II	41.9
Fraction III	38.3
Fraction IV	58.4
Fraction V	75.2
Fraction VI	63.5

*Antioxidative activity is the inhibition percent of superoxide radical at the test concentration of 100 μg/mL. Values of antioxidative activity are expressed as the mean of three experiments.

carbonyl), 1614, 1508, 1429 (aromatic double bond) cm^{-1}; UV: λ_{max} (MeOH) 270, 336 nm, (MeOH + NaOAc) 280, 375 nm, (MeOH + $AlCl_3$) 277, 304, 346 nm, (MeOH + $AlCl_3$ + HCl) 278, 306, 346, 385 nm, (MeOH + NaOMe) 280, 328, 395 nm; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 8.13 (2H, d, J = 8.32 Hz, H-2', 6'), 7.02 (2H, d, J = 8.32 Hz, H-3', 5'), 6.73 (1H, s, H-3), 6.39 (1H, s, H-6), 4.91 (1H, d, J = 9.70 Hz, H-1''); ^{13}C-NMR (75 MHz, DMSO-d_6): δ 183.08 (C-4), 165.02 (C-2), 163.69 (C-7), 162.20 (C-4'), 161.71 (C-5), 157.07 (C-9), 129.67 (C-2', 6'), 122.66 (C-1'), 116.64 (C-3', 5'), 105.42 (C-8), 105.02 (C-10), 103.15 (C-3), 99.01 (C-6), 82.54 (C-5''), 79.92 (C-3''), 74.43 (C-1''), 71.97 (C-2''), 71.60 (C-4''), 62.44 (C-6'').

Measurement of superoxide radical scavenging (NBT test)

Superoxide dismutase (SOD) activity was measured using the xanthine-xanthine oxidase system as a source of superoxides and nitroblue tetrazolium (NBT) as a scavenger for this radical. SOD activity was determined using the methods described by Furuno *et al.* (2002). The inhibition rate of NBT reduced by SOD was measured. In the NBT test, 0.1 mL sample, 2.4 mL Na_2CO_3 buffer (0.05 M, pH 10.2), 0.1 mL xanthine (3 mM), 0.1 mL ethylene diamine tetraacetic acid (EDTA, 3 mM), 0.1 mL NBT solution (0.72 mM) and 0.1 mL bovine serum albumin (BSA) solution were added in order to each test tube and was left to react at 25 °C for 10 min. To each test tube, 0.1 mL xanthine oxidase (0.25 unit/mL) was added, which reacted at 25 °C for 20 min. The reaction was quenched with 0.1 mL $CuCl_2$. Lastly, inhibition (%) was calculated in comparison to the control blank after measuring absorbance at 560 nm.

Measurement of free radical scavenging (DPPH assay)

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical as determined by a radical scavenging assay. In DPPH test, 0.15 mL of the sample and 0.15 mL of DPPH alcoholic solution (0.1 mM) were mixed and incubated in a test plate at 37 °C for 10 min. The absorbance was measured at 560 nm using a microtiter plate reader (Elx800, Bio-Tek Instruments, Vermont, USA). Its antioxidative activity (%) was calculated in comparison to the blank control (Blois, 1958).

Cell culture

Human dermal fibroblasts (HDFs) from newborn foreskin were acquired from Modern Tissue Technology (MTT, Korea). The cells were cultured in DMEM/F12 (3:1) medium with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μg/mL) and incubated in a humidified 5% CO_2 incubator at 37 °C. HDFs from passage 6 to 10 were used in the experiments.

Microtiter plate assay

HDFs (1.5×10^5/mL) were seeded into 96-well plates and cultured overnight. CM-H_2DCFDA, a non-fluorescent compound, is able to react with free radical compounds, especially with hydrogen peroxide, to generate fluorescent DCF. For the detection of ROS, HDFs were loaded with 4.0 μM CM-H_2DCFDA plus 2% Pluronic F-127 in HEPES-buffered control salt solution (HCSS) containing 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES and 10 mM NaOH. The cells were incubated for 20 min at 37 °C to which 20 μL of test sample of varying doses was added. After 30 min at 37 °C the cells (HDFs) were irradiated by a UVB source. UVB irradiation doses were 20 mJ/cm^2 and the radiation intensity was measured by using a UV radiometer (EKO, Japan). Fluorescence was determined using a luminescence spectrophotometer (Perkin Elmer, UK) with an excitation wavelength of 488 nm and emission wavelength of 525 nm (Seo *et al.*, 1999; Trayner *et al.*, 1995; Lee *et al.*, 2003).

Flow cytometry assay

ROS were detected by incubation with CM-H_2DCFDA, which is hydrolyzed intracellularly to H_2DCF, which in turn can be oxidized to the fluorescent DCF by intracellular oxidants (principally hydrogen peroxide). DCF levels in non-irradiated versus irradiated cultures were compared by flow cytometry (FACS Calibur-S System, Becton Dickinson, NJ, USA). HDFs (1.5×10^5/mL) were seeded into 6-well plates and cultured overnight. The cultured fibroblasts were subsequently treated with several doses of test samples. The cells were incubated for 2 h at 37 °C. For the detection of ROS, HDFs were loaded with 1 μM CM- H_2DCFDA plus 2% pluronic F-127 in HCSS containing 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES and 10 mM

NaOH. The cells were incubated for 20 min at 37 °C and HDFs were irradiated by a UVB source. UVB irradiation doses were 20 mJ/cm^2 and the radiation intensity was measured by the UV radiometer.

After cell harvest by trypsinization, the cells were washed twice with HCSS. The fluorescence signal of DCF (Ex = 488 nm; Em = 525 nm), the oxidation product of CM- H_2DCFDA by free radicals, was analyzed by flow cytometry (Seo *et al.*, 1999; Trayner *et al.*, 1995; Lee *et al.*, 2003; Ryoo *et al.*, 2001).

ROS imaging using confocal microscopy

HDFs were treated with several doses of test samples for 2 h, and were irradiated by a UVB source. UVB irradiation doses were 20 mJ/cm^2 and the radiation intensity was measured using UV radiometer.

HDFs grown on a glass-bottom dish were loaded with 2 μM CM-H_2DCFDA plus 2% pluronic F-127 in HCSS containing 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES and 10 mM NaOH. The cells were incubated for 20 min at 37 °C, washed three times with HCSS, and the fluorescence signal of DCF (Ex = 488 nm; Em = 525 nm), i.e. the oxidation product of CM-H_2DCFDA by free radical, was analyzed using Leica DM IRE2 inverted microscope (Leica, Germany). To minimize background signals caused by direct oxidation of CM-H_2DCFDA by illumination at 488 nm, intracellular levels of ROS were analyzed within 3 sec after illumination using a 20° HCX FLUOTA lens/TCS-SP2 confocal system (Leica, Germany).

Statistical analysis

Results were presented as means ± S.E.M. Experimental results were statistically analyzed by using Microsoft EXEL. P values <0.05 were regarded as indicating significant differences.

RESULTS AND DISCUSSION

The ethanolic extract from tinged autumnal leaves of *A. palmatum* was partitioned with n-hexane, $CHCl_3$, ethyl acetate (EtOAc), and BuOH (Table I). Compound **1** obtained from the ethyl acetate fraction of maple leaves showed a dark brown color resulting from the $FeCl_3$ test and yellow color after the chloramin T test. Its R_f value on TLC was 0.74 ($CHCl_3$-ethyl acetate-EtOH=1:1:1). In the IR spectrum, the absorption of the hydroxyl group was shown at 3383 cm^{-1}, and the absorption of the conjugated carbonyl group of the γ-pyron functionally was detected at 1655 cm^{-1} (Ham *et al.*, 1994). The UV λ_{max} absorbances of compound **1** (MeOH) showed a typical pattern of flavones at 270 and 336 nm (Ham *et al.*, 1994). In ^{1}H NMR spectrum, two doublet signals at δ 8.13 (d, *J* = 8.32 Hz, H-2,6) and δ 7.02 (d, *J* = 8.32 Hz, H-3, 5) suggested the typical A_2B_2 splitting pattern of flavonoid ring B. In addition, two singlet signals were detected at δ 6.73 and 6.39, the characteristics of an apigenin moiety missing a peak of H-3 or 8 (Oh *et al.*, 1994) was showed. Because there were only two signals and no splitting, the distance between two protons was expected not to be close. Therefore, the signal of 6.73 ppm is expected from the proton of position 3 of the C ring, and that of 6.39 ppm would be the proton located in position 6 of the A ring. The typical signal of a beta anomeric proton is detected at δ 4.91 (1H, d, *J* = 9.70 Hz, 1) and other protons of D-glucose are detected as a multiplet at 3.40~4.00 ppm. In the ^{13}C-NMR spectrum, carbonyl carbon was shown at 183.10 ppm, and the signals of the fourteen aromatic carbons are detected at δ 99.00 to 165.00. Compound **1** was C-glycoside, and six sp^3 carbon signals of the sugar moiety were confirmed at δ 82.6 (C-5), 79.9 (C-3), 74.4 (C-1), 71.9 (C-2), 71.6 (C-4), and 62.4 (C-6). In addition, there was no reaction upon attempted acid hydrolysis. Therefore, the anomeric carbon of the sugar would be expected to bind directly with the carbon of the flavonoids. From these results, the chemical structure of the isolated compound was identified as apigenin-8-C-beta-D-glucopyranoside, vitexin, on the basis of FT-IR, ^{1}H, ^{13}C-NMR and UV spectral data, and was elucidated based on previously reported data on its structure (Ham *et al.*, 1994; Oh *et al.*, 1994; Yoo *et al.*, 2002; Greenham *et al.*, 2003).

The exposure of cells to UVB radiation is able to induce the production of ROS, which damage cellular components. Free radical scavengers and antioxidants can inhibit the production of ROS. Katiyar *et al.* (2001) reported that green tea polyphenol (-)-epigallocatechin-3-gallate on human skin inhibits ultraviolet radiation-induced oxidative stress. Other investigators reported the correlation of *in vitro* anti-oxidative and *in vivo* photoprotective effects of plant extract (Tobi *et al.*, 2002; Bonina *et al.*, 2002; Bandoniene *et al.*, 2002; Savini *et al.*, 1999). Superoxide radical is one of the strongest free radical in cellular oxidation reactions because it further produces various kinds of cell-damaging free radicals and oxidizing agents (Harman *et al.*, 1997). Free radical production is implicated in many conditions, including heart disease,

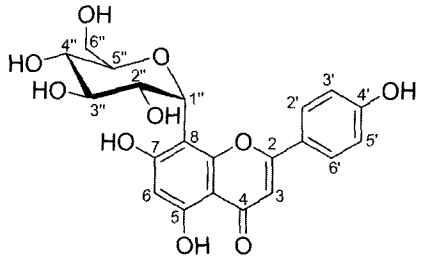

Fig. 1. The structure of vitexin (apigenin-8-C-beta-D-glucopyranoside)

cancer, arthritis, and aging. Fig. 2 presents the results of the NBT test on vitexin compared with other well-known antioxidants to measure superoxide (O_2) scavenging effect. The activity of vitexin was increased in a dose dependent manner. The IC_{50} value calculated from a semi-log scale was 40 μg/mL and BHT was 45 μg/mL. The activity of vitexin was comparatively as good as BHT or higher than BHT, but it seemed to be less effective than EGCG at a concentration of 10 μg/mL.

Free radicals are molecules or parts of molecules produced by the metabolic processes of oxygen. They can evoke a chain reaction causing the multiplication of new free radicals, which can result in interference and manipulation of proteins, tissue loosening, genetic damage and the promotion of disease and aging (Katiyar *et al.,* 2001). Fig. 3 presents the results of the DPPH test on vitexin to measure free radical scavenging effects and validate the correlation with the NBT test. Free radical scavenging activity of vitexin was about 60% at a concentration of 100 μg/mL. The IC_{50} value of vitexin in this test was 84 μg/mL.

UV irradiation produces free radicals and related ROS, which can injure almost all of components of skin cells and the extracellular matrix (ECM). Groving evidence shows that flavonoids are powerful scavengers of ROS, inhibitors of lipid peroxidation, metal ion chelators, and inhibitors of enzymes related to inflammation, *in vivo* and *in vitro* (Bonina *et al.*, 2002; Middleton *et al.*, 2000; Nijveldt *et al.*, 2001). In testing intracellular ROS scavenging

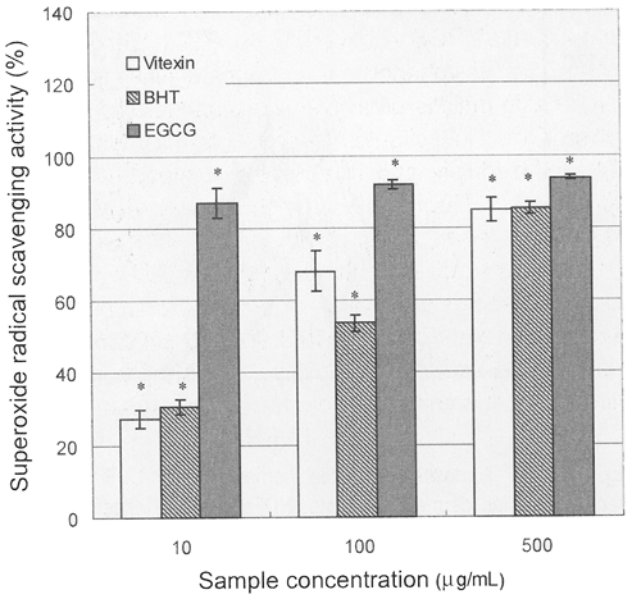

Fig. 2. Effects of superoxide radical scavenging (NBT). BHT and EGCG were used as positive controls. The superoxide radical scavenging activity of vitexin is indicated by percent increase compared with control activity. The activity is significant (*p<0.05) and the values are expressed as mean ± S.E.M.

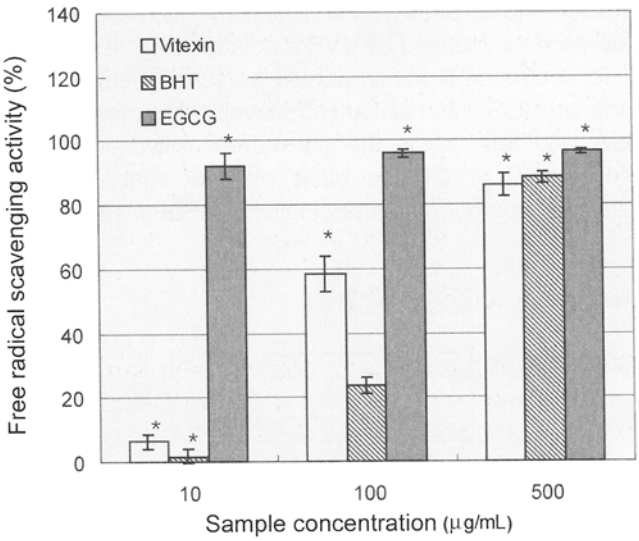

Fig. 3. Effects of free radical scavenging (DPPH). BHT and EGCG were used as positive controls. The free radical scavenging activity of vitexin is indicated by percent increase compared to control activity. The activity is significant (*p<0.05). Values are expressed as mean ± S.E.M.

activity, oxidative stress was also analyzed by determining the increases in DCF fluorescence upon exposure to UVB 20 mJ/cm^2. Savini *et al.* (1999) showed that UVB exposure increased the intracellular content of peroxide as monitored by measuring the fluorescent emission of DCF. Cells exposed to exogenous ascorbic acid were able to completely prevent the increases in intracellular peroxides induced by UVB. Furthermore, Bestwick and Milne (2001) reported that quercetin may contribute to the protection afforded by fruit- and vegetable-rich diets against diseases for which excess production of ROS has been implicated as a casual or contributory factor. In this study, the basal and UVB-induced levels of fluorescence for CM-H_2DCFDA loaded HDFs after vitexin treatment are shown in Fig. 4A. The basal level of ROS in HDFs in the normal culture condition was about 120~160 AU, while that of CM-H_2DCFDA solution was about 50 AU in the same condition ($p < 0.05$). UVB exposure (20 mJ/cm^2) produced an increase of about 2.4 fold over basal levels (fluorescence value: 568 AU) in fluorescence in CM-H_2DCFDA-loaded cells. After treatment with vitexin in the culture medium for 2 h, the value of DCF fluorescence decreased remarkably in a dose dependent manner. Regarding these tests, vitexin treatment up to 1 μg/mL had little effect on the levels of fluorescence in UV-irradiated cells; however, after 5~50 μg/mL of vitexin treatment there was decrease of 25-72% in the levels of fluorescent DCF. Moreover, a concentration of 100 μg/mL concentration of vitexin reduced the UVB-induced fluorescence increase close to baseline value (the corresponding unirradiated level). Comparing to

EGCG, vitexin effectively inhibited the increase in DCF fluorescence induced by UVB irradiation (Fig. 4B).

To determine if vitexin served as scavengers of UVB-induced ROS, intracellular ROS levels were measured by flow cytometer using the peroxide-sensitive fluorescent indicator, DCF. On the basis of laser light scatter, a relatively homogeneous population of HDFs was shown.

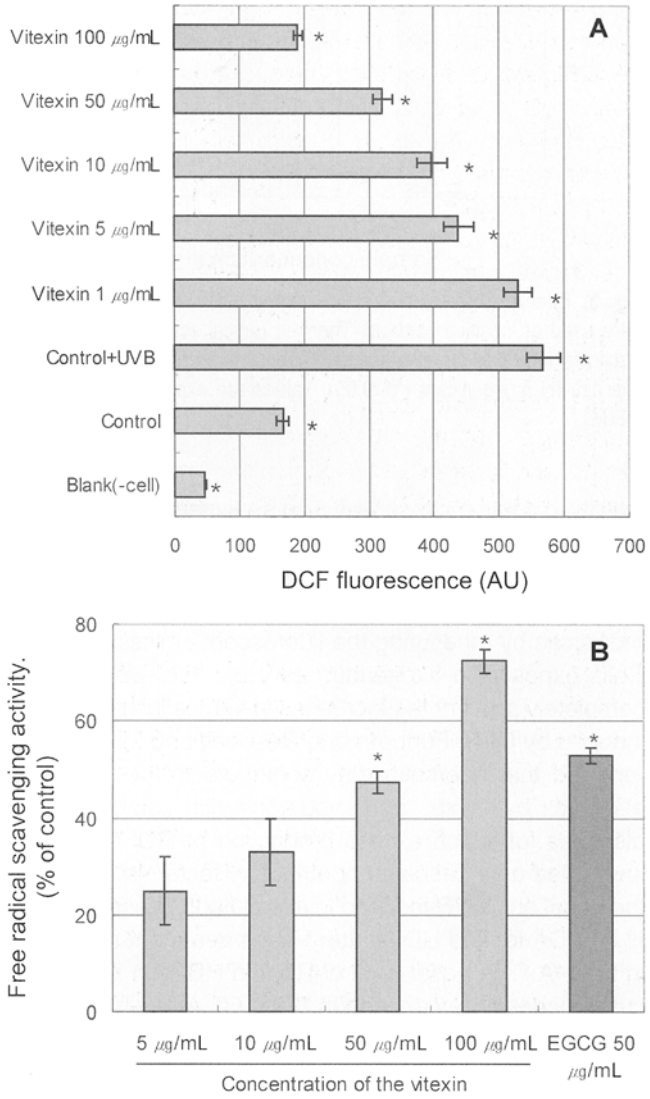

Fig. 4. A: The effects of vitexin on the production of intracellular reactive oxygen species (ROS) in human dermal fibroblasts (HDFs). HDFs were incubated with 4 μM CM-H_2DCFDA for 20 min, and irradiated by UVB 20 mJ/cm^2. Various concentrations of vitexin were used for treatment. ROS generation was assessed by luminescence spectrophotometer. The values of DCF fluorescence are significant (*$p<0.05$). Values are expressed as mean ± S.E.M. B: Free radical scavenging activity after UV irradiation in human dermal fibroblasts (HDFs). ROS generation was assessed by luminescence spectrophotometer. Vitexin (□) reduced the UVB-induced fluorescence increase in a dose dependent manner. EGCG (■) was used as a positive control. The values of DCF fluorescence are significant (*$p<0.05$). Values are expressed as mean ± S.E.M.

The data from flow cytometry showed that individual cells were activated at different times following UVB irradiation (data not shown). In the absence of CM-H_2DCFDA, the cells displayed very low autofluorescence with mean channel intensity <10 AU (data not shown). In the presence of CM-H_2DCFDA, significant fluorescence was detected within these cells with mean channel intensity of 60-80 AU. After UVB irradiation, HDFs showed higher basal levels of ROS (intensity of about 120 AU) compared with the DCF-containing control cells. The peak was shifted to the right and the fluorescence value was higher than the previous peak. In order to measure free radical scavenging activity in UVB-irradiated HDFs, we incubated the cells in the presence of active materials prior to UVB irradiation. A significant inhibition of the intracellular fluorescence was observed by the addition of vitexin and EGCG (50 μg/mL). After the treatment of vitexin and EGCG for 2 h ROS levels decreased by approximately 37% in response to vitexin and 56% in response to EGCG compared with UVB-irradiated control HDFs. These results show that oxidation of CM-H_2DCFDA is effectively inhibited by vitexin and that vitexin has a potent free radical scavenging activity in UVB-irradiated HDFs (Fig. 5).

The study based on H_2DCF oxidation to DCF has often been used to measure intracellular H_2O_2 or oxidative stress (Cathcart *et al.*, 1983; LeBel *et al.*, 1992). H_2DCF is oxidized to DCF by a hydroxy radical, nitrogen dioxide radical (NO^{2-}), and bicarbonate radical anion (Zhang *et al.*,

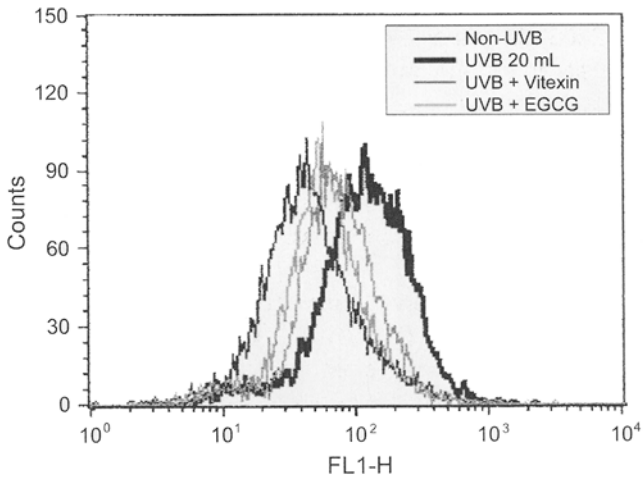

Fig. 5. DCF fluorescence profile comparing non-UVB with UVB irradiated human dermal fibroblasts (HDFs) and the effects of vitexin and EGCG. This panel reports the generation of intracellular ROS after UVB exposure and the effects of vitexin (50 μg/mL) and EGCG (50 μg/mL). HDFs were incubated with vitexin or EGCG in the culture medium for 2 h. After washing the cells twice, they were irradiated by a UVB lamp (UVB 20 mJ) and treated with carboxymethyl-27-dihydro-dichlorofluorescein diacetate (CM-H_2DCFDA, 1 μM). After 20 min the ROS generation was assessed by flow cytometry, using CM- H_2DCFDA as a probe.

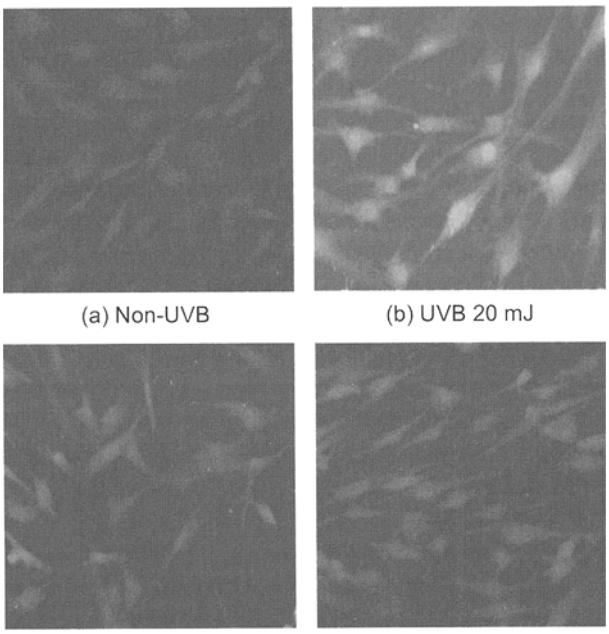

Fig. 6. Confocal microscopic observation of ROS by CM-H_2DCFDA staining in cultured human dermal fibroblasts (HDFs). (a) Control: HDFs under non-UVB conditions, (b) exposed to 20 mJ/cm^2 UVB-irradiation, (c) pretreated with 50 μg/mL EGCG for 2 h, followed by treatment with 20 mJ/cm^2 UVB-irradiation, (d) pretreated with 50 μg/mL Vitexin for 2 h, followed by treatment with 20 mJ/cm^2 UVB-irradiation. Magnification : × 400.

2000). In several studies, H_2DCF was used to monitor intracellular oxidative stress during apoptosis (Tampo *et al.*, 2003). In this study, we observed green fluorescence intensity to determine free radical scavenging activity of vitexin using CM-H_2DCFDA in cultured fibroblasts. In HDFs without UVB exposure, little fluorescence induction was noticed (basal level). Upon UVB irradiation, we observed an induction of ROS to bright green fluorescence in the cultured fibroblasts. By confocal microscope, newly induced ROS appearing in the nucleus and cytoplasm was observed (see the green labeling in Fig. 6). Upon irradiation of UVB 20 mJ/cm^2, the intensity of green fluorescence is higher comparing to non-irradiated cells. After the treatment of vitexin and EGCG at 50 μg/mL, we observed decreases of ROS induction compared to untreated cells after UVB irradiation. Thus, the low intensity of the green fluorescence was highly suggestive of a ROS scavenging effect of vitexin and EGCG. The results showed that vitexin may be photoprotective agents in UV irradiated cells.

REFERENCES

Afitlhile, M. M., Dent, R. M., and Cowan, A. K., Changes in carotenoid composition in senescing leaves of *Hordeum vulgare* L. cv. Dyan. *J. Plant Physiol.*, 142, 4349 (1993).

Aritomi, M., Chemical constituents in Aceraceous plants. 1. Flavonoid constituents in the leaves of *Acer palmatum* Thunberg. *Yakugaku Zasshi*, 83, 737-740 (1963).

Aritomi, M., Chemical constituents in Aceraceous plants. 3. Flavonoid constituents in leaves of *Acer cissifolium* K. Koch. *Chem. Pharm. Bull.*, 12, 841-843 (1964).

Bandoniene, D. and Murkovic, M., On-line HPLC-DPPH screening method for evaluation of radical scavenging phenols extracted from apples (*Malus domestica* L.). *J. Agric. Food Chem.*, 50, 2482-2487 (2002).

Bestwick, C. S. and Milne, L., Quercetin modifies reactive oxygen levels but exerts only partial protection against oxidative stress within HL-60 cells. *Biochim. Biophys. Acta*, 1528, 48-59 (2001).

Blois, M. S., Antioxidant determinations by the use of a stable free radical. *Nature*, 181, 1199-1200 (1958).

Bonina, F., Puglia, C., Ventura, D., Aquino, R., Tortora, S., Sacchi, A., Saija, A., Tomaino, A., Pellegrino, M. L., and de Capariis P., *In vitro* antioxidant and *in vivo* photoprotective effects of a lyophilized extract of *Capparis spinosa* L. buds. *J. Cosmet. Sci.*, 53, 321-335 (2002).

Bors, W. and Michel, C., Chemistry of the antioxidant effect of polyphenols. *Ann. N. Y. Acad. Sci.*, 957, 57-69 (2002).

Cathcart, R., Schwiers, E., and Ames, B. N., Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal. Biochem.*, 134, 111-116 (1983).

Dauborn, B. and Brueggemann, W., A spontaneous point mutation in rubisco large subunit gene impairing holoenzyme assemblyrenders the IV beta plastome mutant of Oenothera extremely light and chilling sensitive. *Physiol. Plant*, 104, 116-124 (1998).

Feild, T. S., Lee, D. W., and Holbrook, N. M., Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood, *Plant Physiol.*, 127, 566-574 (2001).

Furuno, K., Akasako, T., and Sugihara, N., The contribution of the pyrogallol moiety to the superoxide radical scavenging activity of flavonoids. *Biol. Pharm. Bull.*, 25, 19-23 (2002).

Gaitan, E., Cooksey, R. C., Legan, J., and Lindsay, R. H., Antithyroid effects *in vivo* and *in vitro* of vitexin : a C-glycosylflavone in millet. *J. Clin. Endocrinol. Metab.*, 80, 1144-1147 (1995).

Greenham, J., Harborne, J. B., and Williams, C. A., Identification of lipophilic flavones and flavonols by comparative HPLC, TLC, and UV spectral analysis. *Phytochem. Anal.*, 14, 100-118 (2003).

Ham, I. H., Oh, I. S., Whang, W. K., and Kim I. H., Pharmaco-constituents of Korean cultivated Rhubarb leaves-the flavonoids from leaves. *Yakhak Hoeji*, 38, 469-475 (1994).

Harborne, J. B., Nature, Distribution and function of plant flavonoids. *Prog. Clin. Biol. Res.*, 213, 15-24 (1986).

Harman, D., Role of free radical reactions in aging and disease.

J. Geriat. Dermatol., 5, 114-127 (1997).

Katiyar, S. K., Afaq, F., Perex, A., and Mukhtar, H., Green tea polyphenol (-)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. *Carcinogenesis*, 22, 287-294 (2001).

Khettab, N., Amory, M. C., Brigand, G., Bousquet, B., and Combre, A., Photoprotective effect of vitamins A and E on polyamine and oxygenated free radical metabolism in hairless mouse epidermis. *Biochimie*, 70, 1709-1713 (1988).

LeBel, C. P., Ischiropoulos, H., and Bondy, S. C., Evaluation of the probe 2,7-dichlorofluorescein as an indicator of reactive oxygen species formation and oxidative stress. *Chem. Res. Toxicol.*, 5, 227-231 (1992).

Lee, B. C., Bae, J. T., Pyo, H. B., Choe, T. B., Kim, S. W., Hwang, H. J., and Yun, J. W., Biological activities of the polysaccharides produced from submerged culture of the edible Basidiomycete *Grifola Frondosa*. *Enzyme Microb. Technol.*, 6274, 1-8 (2003).

Mathews-Roth, M. M., Carotenoid pigment administration and the delay in development of UVB-induced tumors. *Photochem. Photobiol.*, 42, 35-38 (1983).

Middleton, E. Jr., Kandaswami, C., and Theoharides, T. C., The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.*, 52, 673-751 (2000).

Nijveldt, R. J., Nood, E., Hoorn, D. EC, Boelens, P. G., Norren, K., and Leeuwen, P.AM, Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.*, 74, 418-425 (2001).

Norins, A. L., Free radical formation in the skin following exposure to ultraviolet light. *J. Invest. Dermatol.*, 39, 445-448 (1962).

Oh, I. S., Whang, W. K., and Kim, I. H., Constituents of *Crataegus pinnatifida* val. *psilosa* Leaves (II) Flavonoids from BuOH fraction. *Arch. Pharm. Res.*, 17, 314-317 (1994).

Prabhakar, M. C., Bano, H., Kumar, I., Shamsi, M. A., and Khan, M. S. Y., Pharmacological investigations on vitexin. *Planta medica*, 43, 396-403 (1981).

Riipi, M., Ossipov V., Lempa, K., Haukioja, E., Koricheva, J., Ossipova, S., and Pihlaja, K., Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? *Oecologia*, 130, 380-390 (2002).

Ryoo, Y. W., Suh, S. I., Mun, K. C., Kim, B. C., and Lee, K. S., The effects of the melatonin on ultraviolet-B irradiated cultured dermal fibroblasts. *J. Dermatol. Sci.*, 27, 162-169 (2001).

Savini, I., DAngelo, I., Ranalli, M., Melino, G., and Avigliano, L., Ascorbic acid maintenance in HaCaT cells prevents radical formation and apoptosis by UVB. *Free Radic. Biol. Med.*, 26, 1172-1180 (1999).

Seo, S. Y., Kim, E. Y., Kim, H., and Gwang, B. J., Neuroprotective effect of high glucose agains NMDA, free radical and oxygen-glucose deprivation through enhanced mitochondrial potentials. *J. Neurosci.*, 19, 8849-8855 (1999).

Shibamoto, T., Sulfur-containing heterocyclic compounds with antioxidative activity. *Am. Chem. Soc.*, 564, 247-256 (1994).

Tampo, Y., Kotamraju, S., Chitambar, C. R., Kalivendi, S. V., Keszler, A., Joseph, J., and Kalyanaraman, B., Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial cells. *Circ. Res.*, 92, 56-63 (2003).

Tobi, S. E., Gilbert, M., Paul, N., and McMillan, T. J., The green tea polyphenol, epigallocatechin-3-gallate, protects against the oxidative cellular and genotoxic damage of UVA radiation. *Int. J. Cancer*, 102, 439-444 (2002).

Trayner, I. D., Rayner, A. P., Freeamn, G. E., and Farzaneh, F., Quantitative multiwell myeloid differentiation assay using dichlorodihydrofluorescein diacetate (H_2DCFDA) or dihydrorhodamine 123 (H_2R123). *J. Immunol. Methods*, 186, 275-284 (1995).

Yoo, S. W., Kim, J. S., Kang, S. S., Son, K. H., Chang, H. W., Kim, H. P., Bae, K. H., and Lee, C. O., Constituents of the fruits and leaves of *Euodia danielli*. *Arch. Pharm. Res.*, 25, 824-830 (2002).

Young, A. J., Wellings R., and Britton G., The fate of chloroplast pigments during senescence of primary leaves of *Hordeum vulgare* and *Avena sativum*. *J. Plant Physiol.*, 137, 701-705 (1991).

Zhang, H., Joseph, J., Felix, C., and Kalyanaraman, B., Bicarbonate enhances the hydroxylation, nitration, and peroxidation reactions catalyzed by copper, zinc superoxide dismutase : intermediacy of carbomate anion radical. *J. Biol. Chem.*, 275, 14038-14045 (2000).