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Antioxidant Activities of Phenolic Acids on Ultraviolet Radiation-Induced Erythrocyte and Low Density Lipoprotein Oxidation

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The exposure of mammalian cells to UV light induces various deleterious responses. Some of the major harmful effects are DNA damage, cell membrane peroxidation, systemic immune suppression, and aging acceleration. Reactive oxygen species and free radicals are believed to be largely responsible for some of the deleterious effects of UV upon cells. Typical administration of antioxidants has recently proved to represent a successful strategy for protecting the cells against UV-mediated oxidative damage. The objective of this study was to investigate the inhibitory effect of phenolic acids (caffeic acid, ferulic acid, gallic acid, and protocatechuic acid) on oxidative damage in human erythrocytes and low-density lipoprotein (LDL) induced by UVB radiation. The results revealed that the thiobarbituric acids reactive substances induced by UVB were decreased from 2.78 to 0.12−0.89 nmol MDA/mg protein in erythrocyte ghost and from 0.72 to 0.14−0.43 nmol MDA/mg protein in LDL by the addition of phenolic acids (100 μM). Caffeic acid, ferulic acid, and gallic acid exhibited over 85 and 60% inhibitory effect toward UVB-induced oxidation in erythrocytes and LDL, respectively. Phenolic acids, especially gallic acid, could maintain the normal glutathione levels and glutathione peroxidase activity in hemolysate from erythrocytes that were exposed to UVB radiation in comparison with untreated control. The results indicate that the antioxidant activities of caffeic acid and ferulic acid play a potential role in protection against UVB oxidative damage to human erythrocytes and LDL.

KEYWORDS: Ultraviolet B; human erythrocytes; low-density lipoprotein; phenolic acids

INTRODUCTION

UV irradiation is well-known to induce photodamage and to promote premature skin aging, erythema, inflammation, immunodepression, and photocarcinogenesis (*1*). UV light can damage many tissue components including membrane phospholipids, proteins, and nucleic acids. The membranes of mitochondria and erythrocytes are easily oxidation induced by photodynamics and free radicals (*2*). UVB photons could penetrate the capillary plexus in the dermis and interact directly with erythrocytes, resulting in photohemolysis (*3*). The risk of photolytic damage to erythrocytes can be increased by the presence of lipid hydroperoxides from UVB-peroxidized low-density lipoproteins (LDL) (*4*). In recent years, accumulated evidence has demonstrated that UV-induced oxidative damage occurs through the formation of free radicals and reactive oxygen species (ROS), which damage cellular components (*5*). Many studies also showed that the increase of skin exposure to solar UV light could decrease the activity of antioxidant enzymes and the content of antioxidant substances (*6*, *7*). Cejkova et al. (*8*) indicated that UVB radiation changed the activity of catalase, superoxide dismutase (SOD), and xanthine oxidase in corneal epithelium. UV radiation also induced nitride oxide formation in keratinocytes and then caused erythema and inflammation (*9*). Therefore, antioxidants can be used to protect the cells from UV-induced cellular damage by scavenging on free radicals and ROS. Eberlein-Konig et al. (*10*) demonstrated that UVB-mediated phototoxic lysis of human erythrocytes was reduced after oral intake of ascorbic acid and α-tocopherol. The application of antioxidant polyphenols isolated from green tea could afford protection against the carcinogenic effects of UVB radiation (*11*).

Phenolic compounds are widely distributed in various vegetables and fruits and show many physiological and pharmacological functions (*12*, *13*). Many studies have shown that natural polyphenolic compounds in plants could inhibit lipid oxidation induced by free radicals and protect photooxidation (*14*, *15*). In general, monophenols are less efficient as free

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radical scavengers than polyphenols. However, simple phenolic acids are present in many dietary phytochemicals, which are at higher concentrations than the polyphenolic flavonoids. The antioxidant capacity of phenolic acids was influenced by the numbers and the *o*, *p*, or *m* sites of the hydroxyl group on the benzoic acid. Besides the strong effects on scavenging free radicals and antioxidant capacity, phenolic acids also exhibited antiinflammation, antiallergic, antimutation, and inhibition on cardiovascular diseases (*16*, *17*). Lodovici et al. (*18*, *19*) reported that phenolic acids, such as protocatechuic and 4-coumaric acid, exert antioxidant activity in vitro and in vivo and protect the oxidative damage induced by UVB in corneal cells.

UV solar light can pass through the derma and then induce the hemolysis or oxidation of erythrocytes, resulting in inflammation and the formation of erythema. It also could increase the photosensitive reaction and induce oxidative damage of LDL (*4*). However, little is known about the protective effects of phenolic compounds toward UVB irriadiation. Phenolic acids such as caffeic acid (CA), ferulic acid (FA), gallic acid (GA), and protocatechuic acid (PCA) exist in many dietary plants. In this study, we investigated the effects of CA, GA, FA, and PCA on UVB-induced oxidative damage in erythrocytes and photodynamic reactions in LDL.

MATERIALS AND METHODS

Materials. 1,1,3,3-Tetramethoxypropane-5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), butylated hydroxytoluene (BHT), 1-chloro-2,4-dinitro-benzene (CDNB), glutathione (GSH), β-nicotinamide adenine dinucleotide phosphate (β-NADPH), bovine serum albumin (BSA), PCA, CA, GA, and FA were purchased from Sigma Chemical Co. (St. Louis, MO). A Tris and protein assay kit was purchased from Bio-Rad Co. (Hercules, California). Ethylenediaminetetraacetic acid (Na_2-EDTA) was purchased from Showa Co. (Tokyo, Japan).

Scavenging Effects on DPPH Radical. The scavenging effects of phenolic acids on DPPH radical were estimated according to the method of Shimada et al. (*20*). Phenolic acid in 4 mL of water was added to a 1 mL solution of α,α -diphenyl-β- picrylhydrazyl (DPPH) in methanol. The final concentration of DPPH was 0.2 mM. The mixture was shaken vigorously and was allowed to stand for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (U-3000, Hitachi).

Preparation of Erythrocytes, Hemoglobin-Free Ghost, and LDL. Fresh blood was obtained from healthy adult males by venipuncture and collected in tubes containing citrate phosphate dextrose adenine solution (CAPD-1). Blood was centrifuged at 3000 rpm for 10 min at 4 °C. The plasma and the buffy coat were removed by aspiration, and the erythrocytes were washed three times with phosphate-buffered saline (PBS). The erythrocytes were resuspended in 10 volumes of PBS and were used in each experiment.

Hemoglobin-free ghost was prepared according to the methods of Tsuda et al. (*21*) and Virgili et al. (*22*) with some modification. Washed erythrocytes were hemolyzed in 40 volumes of 5 mM phosphate buffer solution (pH 7.4) and centrifuged at 15000 rpm for 20 min at 4 °C. The pellet was washed repeatedly until colorless ghosts were obtained. The hemolysate was collected and used in the experiments.

LDL (d 1.019−1.063 g/mL) was isolated from plasma by density gradient ultracentrifugation using a Hitachi centrifuge (Himac CS 120 GX, Hitachi) as described by Yamanaka et al. (*23*). After isolation, LDL was dialyzed against PBS (10 mM, pH 7.4) containing 10 μM EDTA at 4 °C. Protein was measured using a Bio-Red kit, with BSA as a standard.

UVB Radiation of Erythrocytes and LDL. The UVB radiation was applied at a wavelength of 280−350 nm (peak of emission near 302 nm) using a fluorescent tube (UVP CL-1000: UVP, Inc., CA). The radiation intensity was measured with a UVX Radiometer (UVP CL-1000: UVP, Inc., CA). Erythrocyte suspensions at 10% hematocrit were preincubated with or without different concentrations (10, 50, and 100 μM) of phenolic acids for 4 h. Then, they were treated by UVB radiation for 0, 30, 60, and 120 min. After the UVB radiation, erythrocytes were collected to prepare the hemoglobin-free ghost and hemolysate according to the methods as described above. The GSH content and thiobarbituric acid reactive substances (TBARS) in hemoglobin-free ghost and GSH content and intracellular enzyme activities including glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione S-transferase (GST) in hemolysate were measured, respectively.

LDL solutions (200 μg protein/mL) were incubated with or without various concentrations (10−100 μM) of phenolic acids for 30 min. Then, they were treated by UVB radiation for 0−480 min. After the UVB radiation, the TBARS and relative electrophoretic mobility (REM) of LDL were determined.

Determination of TBARS in Erythrocyte and LDL. The method for determination of TBARS in erythrocyte and LDL was referred from Buege and Aust (*24*) and Yagi (*25*). After radiation, 1 mL of 10% erythrocyte suspension solution or 100 μL of LDL (100 μg protein/mL) was added to BHT (the final concentration was 0.5 mM). Two milliliters of 7.5% TCA was added to the mixture to precipitate protein. The upper layer was drawn, and 1 mL of 1% TBA was added to combine with malonaldehyde (MDA), and the fluorescence at 515 nm excitation and 555 nm emission was determined. 1,1,3,3-Tetramethoxypropane was used as a standard to convert the MDA content.

Determination of GSH Content in Erythrocyte. After radiation, 0.5 mL of erythrocyte ghost or hemolysate solution was added to 2 mL of 5% TCA in ice for 5 min and then centrifuged. The supernatant was mixed well with Tris/EDTA buffer (pH 8.9), and then, DTNB was added and reacted for 5 min. The GSH levels were measured by spectrophotometer (U-3000, Hitachi) at 412 nm and were calculated by $E_{412} = 13600\ M^{-1}\ cm^{-1}$.

Determination of GPx in Erythrocyte. The GPx activity was determined spetrophotometrically according to the method of Lawrence and Burk (*26*). After radiation, 0.1 mL of 10% erythrocyte suspension solution was added to 0.8 mL of 100 mM potassium phosphate buffer (pH 7.4), containing 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/mL GR, and 1 mM GSH. After 5 min, 0.1 mL of 2.5 mM H_2O_2 was added to start the reaction. The changes of absorbance for 3 min were recorded by the spectrophotometer (U-3000, Hitachi) at 340 nm. The enzyme activity was counted by $E_{340} = 6220\ M^{-1}\ cm^{-1}$ and was expressed by nmol NADPH/min/mg protein.

Determination of GR in Erythrocyte. The GR activity was determined according to Bellomo et al. (*27*). After radiation, 0.1 mL of 10% erythrocyte suspension solution was added to 0.9 mL of 100 mM potassium phosphate solution (pH 7.0), containing 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH. The reaction mixture was reacted for 3 min at room temperature, and then, the changes of absorbance for 3 min were recorded by the spectrophotometer (U-3000, Hitachi) at 340 nm. The enzyme activity was measured by $E_{340} = 6220\ M^{-1}\ cm^{-1}$ and was expressed by nmol NADPH/min/mg protein.

Determination of GST in Erythrocyte. The GST activity was determined according to the method of Habig et al. (*28*). After radiation, GST activity was measured using 0.1 mL of 10% erythrocyte suspension solution, 100 mM GSH, and 1 mM CDNB as substrate in a final volume of 1 mL of 0.1 M potassium phosphate buffer (pH 6.5). After it was mixed well, the changes of absorbance for 3 min were recorded by the spectrophotometer (U-3000, Hitachi) at 340 nm. The enzyme activity was counted by $E_{340} = 9.6\ mM^{-1}\ cm^{-1}$ and was expressed by nmol CDNB-GSH conjugated formed/min/mg protein.

Determination of REM of LDL. The REM of LDL was determined using the commercial kit (Sebia HYDRAGEL 7 LDL/HDL Direct ref 4109, Sebia co. France). The method was modified from Sebia HYDRASYS1211 automation process. After electrophoresis, the gel was fixed for 10 min and dried completely. The dried gel was stained for 10 min and then destained. The REM is defined as the ratio of migrating distance of oxidized LDL to that of native LDL.

Statistical Analysis. All analyses were run in triplicate and averaged. Statistical analyses were performed according to the SAS Institute User's Guide. Analyses of variance (ANOVA) were performed using the ANOVA procedure. Significant differences ($P < 0.05$) between the means were determined using Duncan's multiple range test.

Table 1. Concentration of Phenolic Acids for 50% Inhibition (IC_{50}) of Scavenging Effect on DPPH Radical

phenolic acids	DPPH radical scavenging effect IC_{50} (μM)[a]
CA	43.10 ± 2.58 b
FA	51.11 ± 3.13 a
GA	34.95 ± 1.69 c
PCA	43.29 ± 2.15 b

[a] The data represent the means ± SD of three determinations. Values in a column with the same superscripts are not significantly different ($p > 0.05$).

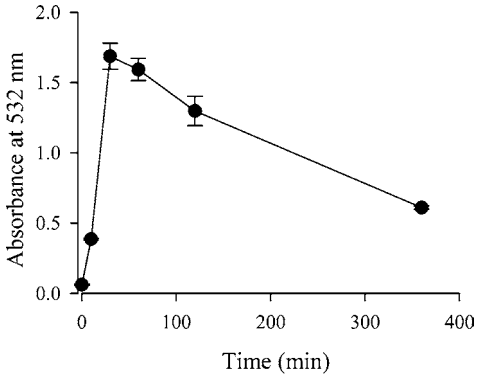

Figure 1. TBARS formation in erythrocyte ghost after various exposure times by UVB.

RESULTS AND DISCUSSION

Scavenging Effects of Phenolic Acids on DPPH Radical. As shown in **Table 1**, the scavenging effects of four phenolic acids on DPPH radical decreased in the order of GA > CA = PCA > FA. The results indicated that the naturally occurring phenolic acids scavenged DPPH radical in a way strongly dependent on substitution pattern and the number of hydroxyl groups on the phenol ring. The inductive effect of the three hydroxyl groups in GA is an important factor that enhances its activity (*29*). Replacing one hydroxy group with a hydrogen atom or methoxy group decreased the radical scavenging effects.

Effects of Phenolic Acids on the Oxidation of Erythrocytes. Figure 1 shows the formation of TBARS in erythrocyte ghost with various exposure times by UVB. The TBARS level in erythrocyte ghost increased with UVB exposure time (0−30 min) up to a maximum and then decreased with the UVB exposure time (60−360 min). This result was similar to the data of Lee et al. (*30*) that MDA level in the rat skin was increased up to 6 h after UVB radiation and then kept decreasing until 48 h. The effects of phenolic acids on the oxidation of erythrocyte ghost are shown in **Table 2**. The formation of TBARS was decreased with increasing the concentration of phenolic acids. The antioxidant activities of phenolic acids toward UVB-induced oxidation of erythrocyte ghost were different from the results of the scavenging effects on DPPH radical. GA showed a stronger inhibitory effect than that of the other three phenolic acids only at a low concentration of 10 μM. However, the inhibitory effects of four phenolic acids decreased in the order of CA > GA > FA > PCA at higher concentrations. The TBARS level of erythrocyte ghost induced by UVB was almost suppressed by addition of 100 μM CA. These results indicated that hydroxycinnamic acid structure is an important factor that increased the antioxidant activity of phenolic acids in erythrocyte membrane. The hydroxycinnamic acid derivatives (CA and FA) exhibited more lipophilicity and presented stronger protection than benzoic acid derivatives (GA and PCA) against membrane lipid peroxidation at higher concentrations, which provided enough hydrogen-donating effects.

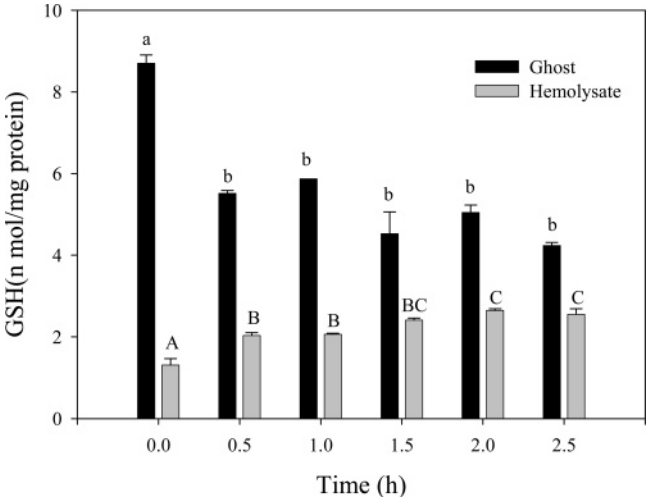

Figure 2. GSH content in erythrocyte ghost and hemolysate after exposure to UVB with various times. Values with differing alphabetic superscripts are significantly different from each other ($p < 0.05$).

Effects of Phenolic Acids on the GSH Levels in Erythrocytes. The changes of GSH levels in ghost and hemolysate from erythrocytes after various exposure times by UVB are shown in **Figure 2**. The GSH content in erythrocyte ghost was significantly decreased when erythrocytes exposed to UVB radiation for 30 min. However, there was no significantly change in GSH content at longer exposure periods, i.e., 1−2.5 h. In contrast, the GSH content in hemolysate was increased with increasing UVB exposure time. Carini et al. (*31*) reported that UVB caused a significant and dose-dependent consumption of intracellular GSH after erythrocytes were exposed to UVB radiation. However, Misra et al. (*32*) indicated that the decrease of GSH content in erythrocyte ghosts at a low dose of UVB exposure was found to be recovered at a higher dose. The changes of GSH contents in erythrocyte ghost and hemolysate may be influenced by the complete structure of the membrane and the action of antioxidant enzymes such as GPx and GR.

Table 2. Effects of Phenolic Acids on the Formation of TBARS in Erythrocyte Ghost Induced by UVB Radiation

treatments[a]	TBARS (MDA nmol/mg protein)[b] CA	FA	GA	PCA
control	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
UVB radiation	2.78 ± 0.12 a	2.78 ± 0.12 a	2.78 ± 0.12 a	2.78 ± 0.12 a
UVB radiation + 10 μM phenolic acid	2.06 ± 0.09 b	2.08 ± 0.26 b	1.76 ± 0.14 b	2.36 ± 0.28 b
UVB radiation + 50 μM phenolic acid	0.24 ± 0.04 c	0.73 ± 0.02 c	0.59 ± 0.06 c	1.32 ± 0.08 c
UVB radiation + 100 μM phenolic acid	0.12 ± 0.01 d	0.36 ± 0.02 d	0.30 ± 0.03 d	0.89 ± 0.07 d

[a] Erythrocyte suspensions at 10% hematocrit were preincubated with or without various concentrations (10, 50, and 100 μM) of phenolic acids for 4 h. Then, they were treated by solar radiation of UVB for 30 min. [b] The data represent the means ± SD of three determinations. Values in a column with the same superscripts are not significantly different ($p > 0.05$).

Table 3. Effects of Phenolic Acids on GPx Activity in Erythrocyte Exposure to UVB Radiation

treatments[a]	GPx activity (nmol/min/mg protein)[b]
control	0.47 ± 0.09
UVB radiation	0.19 ± 0.04 b
UVB radiation + 100 μM CA	0.40 ± 0.13 a
UVB radiation + 100 μM FA	0.35 ± 0.14 a
UVB radiation + 100 μM GA	0.46 ± 0.10 a
UVB radiation + 100 μM PCA	0.40 ± 0.03 a

[a] Erythrocyte suspensions at 10% hematocrit were preincubated with or without of phenolic acids for 4 h. Then, they were treated by solar radiation of UVB for 30 min. [b] The data represent the means ± SD of three determinations. Values in a column with the same superscripts are not significantly different ($p > 0.05$).

Table 4. Effects of Phenolic Acids on the Formation of TBARS in LDL Induced by UVB Radiation

treatments	TBARS (nmol MDA/mg protein)[a]
control	0.028 ± 0.003
UVB radiation	0.720 ± 0.089 a
UVB radiation + 10 μM CA	0.365 ± 0.012 d
UVB radiation + 100 μM CA	0.203 ± 0.017 f
UVB radiation + 10 μM FA	0.173 ± 0.115 fg
UVB radiation + 100 μM FA	0.144 ± 0.005 g
UVB radiation + 10 μM GA	0.528 ± 0.016 b
UVB radiation + 100 μM GA	0.264 ± 0.004 e
UVB radiation + 10 μM PCA	0.635 ± 0.019 a
UVB radiation + 100 μM PCA	0.431 ± 0.009 c

[a] The data represent the means ± SD of three determinations. Values in a column with the same superscripts are not significantly different ($p > 0.05$).

The effects of phenolic acids on the GSH content in erythrocytes were also measured. Phenolic acids, especially GA (10 μM), could maintain the normal GSH levels (1.37 ± 0.08 nmol GSH/mg protein) in hemolysate from erythrocytes that were exposed to UVB radiation (2.03 ± 0.16 nmol GSH/mg protein) in comparison with untreated control (1.31 ± 0.37 ± 0.08 nmol GSH/mg protein). However, they did not prevent the GSH loss in erythrocyte ghosts (data not shown). Carini et al. (*31*) also showed that procyanidins did not suppress GSH depletion at any dose of UVB radiation.

Effects of Phenolic Acids on the Antioxidant Enzymes in Erythrocytes. Table 3 summarizes the GPx activity in erythrocytes treated by UVB with or without the presence of phenolic acids. Phenolic acids could inhibit the inactivity of GPX induced by exposure to UVB radiation. However, there were no significant differences in the activities of GST and GR when erythrocytes were treated with UVB in either the presence or the absence of phenolic acids in comparison to control (data not shown). Lee et al. (*30*) indicated that catalase, GPx, and GR in rat skin were shown to lose their activities easily by UVB radiation, while SOD was relatively stable to the large dose of UVB. Seo et al. (*33*) also demonstrated that chronic UVB exposure caused a decrease in GST activity of the skin tissue. GST, GPx, and SOD may play an important role in protecting skin from UV radiation; however, the study on the response of antioxidant enzymes in erythrocytes to UV radiation is limited at present.

Effects of Phenolic Acids on the Oxidation of LDL. As shown in **Table 4**, the TBARS levels of LDL were increased by exposure LDL to UVB radiation. However, the addition of phenolic acids to LDL could significantly decrease the TBARS content of LDL in the order of FA > CA > GA > PCA. Laranjinha et al. (*34*) indicated that the inhibition of phenolic acids on the metmyoglobin/hydrogen peroxide-dependent LDL peroxidation decreased in the order of CA ≥ chlorogenic acid ≥ PCA > FA > ellagic acid. Sánchez-Moreno et al. (*29*) also reported that the protection of phenolic acids toward oxidation of LDL induced by Cu^{2+} decreased in the order of tannic acid ≥ CA ≥ GA > FA. CA had a stronger protection on the oxidation of LDL, which was induced by hydrophilic prooxidants. However, in our results, FA exhibited a more inhibitory effect against UVB-induced oxidation of LDL. Saija et al. (*35*, *36*) indicated that CA and FA could permeate through the stratum corneum and afford a significant protection to skin against UVB-induced erythema. FA showed greater photoprotection; this might be due to its higher lipophilicity and its easier permeability through the stratum corneum than CA. Sánchez-Moreno et al. (*29*) also demonstrated that the inhibition of linoleic acid autoxidation followed the order of FA > tannic acid = GA > CA. Although CA and FA have relatively similar structures, their antioxidant activities are different. The presence of a methoxy group in FA could increase lipophilicity and could be the key factor for the inhibition of lipid oxidation.

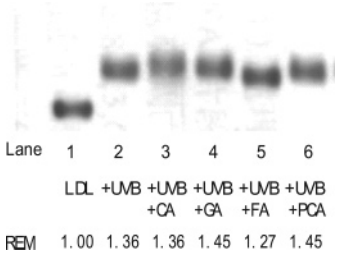

Figure 3. Effects of phenolic acids (100 μM) on the REM of LDL (200 μg/mL) after UVB radiation for 4 h.

The REM of LDL after exposure to UVB radiation was also measured (**Figure 3**). Except the FA, the other three phenolic acids (100 μM) did not change the REM of LDL, which was exposed to UVB radiation. Phenolic acids exhibited strong inhibitory effects on the lipid peroxidation of LDL. However, they could not protect against the damage of the protein portion contained in LDL induced by UVB. Khalil et al. (*37*) indicated that the depletion of vitamin E correlated well with the formation of conjugated dienes and TBARS observed when LDL was exposed to ionizing radiation. Moreover, the susceptibility of LDL to oxidation induced by ionizing radiation increased with age (*38*).

In conclusion, on the basis of the results of the present study, the phenolic acids were found to possess a strong antioxidant activity against peroxidation in erythrocytes and LDL induced by UVB radiation. The antioxidant abilities of phenolic acids varied with their lipophilicity and hydrogen-donating effects. The free radical scavenging effects of phenolic acids were dependent with their numbers of hydroxy groups. However, the antioxidant activities of phenolic acids in erythrocyte membrane and LDL were influenced by the remaining molecular structure. The hydroxycinnamic acid derivatives (CA and FA) exhibited more lipophilicity and presented stronger protection than benzoic acid derivatives (GA and PCA) against membrane lipid peroxidation, especially at higher concentrations. Therefore, phenolic acids can be used for a variety of beneficial chemopreventive effects toward the damage of UVB radiation.

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