Metabolism of Antioxidant in Lemon Fruit (*Citrus limon* BURM. f.) by Human Intestinal Bacteria

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The metabolism of eriocitrin (eriodictyol 7-rutinoside), an antioxidant in lemon fruit (*Citrus limon* BURM. f.), was examined using intestinal bacteria. In the experiment, it was shown that eriocitrin was hydrolyzed to eriodictyol, its aglycon, by *Bacteroides distasonis* and *Bacteroides uniformis*. Eriodictyol was converted to 3,4-dihydroxyhydrocinnamic acid (3,4,-DHCA) and phloroglucinol (PHL) by *Clostridium butyricum*. The metabolic pathway of eriocitrin to eriodictyol and 3,4-DHCA was confirmed by using human feces. The antioxidative activity of eriocitrin metabolites, eriodictyol, 3,4-DHCA, and PHL, was examined using linoleic acid autoxidation and a rabbit erythrocyte membrane oxidation system. It was shown that eriodictyol exhibited stronger activity than α-tocopherol. The activities of 3,4-DHCA and PHL were weak, but they preserved the antioxidative activity.

Keywords: *Lemon fruit; Citrus limon; antioxidant; eriocitrin; flavanone; metabolism; intestinal bacteria*

INTRODUCTION

Lipid peroxidation is known as one of the major factors in the deterioration of food during storage and processing. In addition, it is thought that lipid peroxidation is strongly associated with aging and carcinogenesis (Yagi, 1987; Harman, 1982; Cutler, 1984). Recently, it was reported that dietary antioxidants may offer effective protection from peroxidative damage in living systems and may play an important role in the prevention of carcinogenesis and in extending the life span of animals (Cutler, 1984, 1992; Osawa et al., 1990). Therefore, much attention has been focused on natural antioxidants, and some polyphenol and β-diketone types of antioxidants have been isolated from natural sources with high antioxidative activity (Tuda et al., 1994; Katsuzaki et al., 1993; Osawa et al., 1992; Nishina et al., 1992). These antioxidants in foods are expected to provide antioxidative activity *in vivo* and protection from peroxidative damage in living systems related to aging and carcinogenesis. To investigate this point, it is important to determine how antioxidants in food are metabolized *in vivo* and how antioxidant metabolites function in a living system. For example, the antioxidative mechanism of curcumin in turmeric *in vivo* was reported (Osawa et al., 1995; Sugiyama et al., 1996).

Lemon fruit (*Citrus limon* BURM. f.) is used in various food preparations, such as soft drinks, alcoholic drinks, and jams. Lemons contain a number of nutrients such as citric acid, ascorbic acid, minerals, and flavonoids. Flavonoid compounds are widespread in the plant kingdom and comprise a large group of naturally occurring compounds found in all vascular plants. They are present in citrus as well as in other fruits, vegetables, nuts, seeds, grains, tea, and wine. The average daily Western diet contains ~1 g of mixed flavonoids (Kuhnan, 1976). Flavonoids that exhibit beneficial effects on capillary permeability and fragility were once known as vitamin P. The flavonoids in citrus fruit had been investigated regarding their physiological function (Middleton and Kandaswami, 1994; Matsubara et al., 1985).

Recently, we have been involved in the isolation of antioxidative compounds from lemons and have identified eriocitrin (eriodictyol 7-rutinoside) from a flavonoid glycoside (Miyake et al., 1997). It was found to be present mainly in lemons and limes. It is important to determine how eriocitrin is metabolized *in vivo* to examine its antioxidative effect in a living system. The metabolites of rutin, a dietary flavonoid, exist in many vegetables and fruits, and it has been reported that rutin is metabolized to quercetin, phloroglucinol (PHL), and 3,4-dihydroxyphenylacetic acid by human intestinal bacteria (Winter et al., 1989). Also, hydroxyphenylacetic and phenylpropionic acids were detected in urine after the ingestion of rutin in rats (Booth et al., 1956). However, the antioxidative mechanism and metabolites of eriocitrin are not yet clear. In this study, we explored the metabolic pathway of eriocitrin by intestinal bacteria cultured from human feces. Also, we examined the antioxidative activity of eriocitrin metabolites.

MATERIALS AND METHODS

Materials. Linoleic acid and α-tocopherol (Toc) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. 3,4-Dihydroxyhydrocinnamic acid (3,4-DHCA) was purchased from Aldrich Chemical Co., Milwaukee, WI. Flavonoid compounds and phenolic compounds were purchased from Funakoshi, Ltd., Tokyo, Japan. Eriocitrin was purified from lemon peel extract using preparative HPLC (Miyake et al., 1997). The intestinal bacteria were supplied from the Japan Collection of Microorganisms (JCM), RIKEN, Japan, the Institute for Fermentation (IFO), Osaka, Japan, and the Institute of Applied Microbiology (IAM), University of Tokyo, Japan.

Media and Culture Method of Bacteria. The medium of *Bifidobacterium* (*Bifidobacterium adolescentis* JCM 1275,

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Bifidobacterium bifidum IFO 14252) was prepared with casein peptone tryptic digest (10 g), meat extract (5 g), yeast extract (5 g), glucose (10 g), K_2HPO_4 (10 g), Tween 80 (1.0 mL), and distilled water (1 L); pH was adjusted to 6.8. Successively, after 10 mL of the medium was sterilized at 121 °C for 15 min, a solution of sodium ascorbate and cysteine-HCl was aseptically added to final concentrations of 1.0% and 0.05%, respectively. The media for *Bacteroides* (*Bacteroides uniformis* JCM 5828, *Bacteroides distasonis* JCM 5825), *Propionibacterium* (*Propionibacterium acnes* ATCC 11827), *Lactobacillus* (*Lactobacillus acidphilusm*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus leichmanii*, *Lactobacillus plantarum* IAM 12477), and *Streptococcus* (*Streptococcus faecalis* IFO 12964, *Streptococcus lactis*) used GAM semisolid (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and that of *Clostridium* (*Clostridium butyricum* IFO 13949, *Clostridium spiroforme* JCM 1432, *Clostridium paraputificum* JCM 1293, *Clostridium perfringens* JCM 3817) used TGC medium (Eiken Chemical Co., Ltd., Tokyo, Japan). The medium of *Enterobacter* (*Enterobacter cloacae* IAM 12349) used NB medium (Nissui Pharmaceutical), and that of *Enterococcus* (*Enterococcus faecalis* IFO 12970) used Typtosoya medium (Nissui Pharmaceutical). These media were sterilized at 121 °C for 15 min and cooled to room temperature. *Bifidobacterium*, *Bacteroides*, *Propionibacterium*, *Lactobacillus*, and *Clostridium* were inoculated anaerobically into 10 mL of their respective media under N_2 gas atmosphere and cultured at 37 °C for 2 days under anaerobic conditions in Gas Pack jars (BBL Microbiology Systems, Becton Dickinson Overseas, Inc., Tokyo, Japan). *Streptococcus*, *Enterobacter*, and *Enterococcus* were aseptically inoculated into their respective media and incubated at 37 °C for 2 days under aerobic conditions.

Metabolism of Eriocitrin by Intestinal Bacteria. Intestinal bacteria were cultured in 10 mL of their respective media for 24 h. To the media (10 mL) were added 20 μL of 50 mg/mL eriocitrin dissolved in water and 50 mg/mL eriodictyol dissolved in dimethyl sulfoxide. Dimethyl sulfoxide (0.2%) did not interfere with the bacterial growth. The media were inoculated with 0.1 mL of precultured medium and were cultured for 2 or 3 days. The metabolism of eriocitrin and eriodictyol was analyzed by HPLC. The decrease ratio of eriocitrin and eriodictyol was determined from the peak area before and after culture for HPLC analysis. HPLC (LC-10A, Shimadzu Co., Ltd., Kyoto, Japan) was carried out using a Shim-pack CLC-ODS(M) (4.6 $\times$ 250 mm, Shimadzu) with a UV detector (280 nm). The mobile phase for HPLC was a gradient linear concentration of 5% acetic acid from 90% to 10% and methanol from 10% to 90% for 40 min at a flow rate of 1 mL/min.

Identification of Eriocitrin Metabolites. The eriocitrin metabolites were purified by preparative HPLC (LC-8A, Shimadzu). Preparative high-performance liquid chromatography (HPLC) was carried out using a YMC-Pack ODS column (YMC Co., Ltd., Kyoto, Japan, 20 $\times$ 250 mm) with a UV spectrophotometric detector (280 nm) and 60% of a 5% acetic acid solution and 40% methanol as the solvent at a flow rate of 10 mL/min. The eriocitrin metabolites were analyzed by GC/MS (Hewlett-Packard HP 6890 Series) and ^{1}H NMR and ^{13}C NMR (JEOL JNM-EX-270 NMR). GC/MS was carried out using a capillary column (HP-5 cross-linked 5% phenyl methyl silicone, 0.25 mm $\times$ 30 m; film thickness, 0.25 mm; Hewlett-Packard Co., Palo Alto, CA) with an injection temperature of 275 °C, a detection temperature of 325 °C, and a column oven temperature program from 80 to 230 °C at 12 °C/min. ^{1}H NMR and ^{13}C NMR spectra were obtained using a JEOL JNM-EX-270 NMR instrument (270 MHz for ^{1}H and 67.5 MHz for ^{13}C) in CD_3OD-*d* containing tetramethylsilane (TMS) as the internal standard.

Treatment of Eriocitrin by Human Feces. Briefly, fecal samples from three healthy volunteers were collected in stool cups. Within 30 min, about 0.1 g of fresh human feces was incubated in 10 mL of brain heart infusion medium (BHI medium, Nissui Pharmaceutical) in Gas Pack jars at 37 °C for 24 h (MacDonald et al., 1983; Mazaki et al., 1982). One milliliter of the culture medium was anaerobically inoculated into 10 mL of the medium containing 1 mg/mL eriocitrin under N_2 gas atmosphere and cultured at 37 °C for 2 days under anaerobic conditions in Gas Pack jars.

Antioxidative Assay of Linoleic Acid Autoxidation Model. Antioxidative activity was evaluated using the linoleic acid system (Osawa and Namiki, 1981). Each sample was added to a solution mixture of linoleic acid (0.13 mL), 99.0% ethanol (10 mL), and a 50 mM sodium phosphate buffer (pH 7.0, 10 mL), and the volume was adjusted to 25 mL with distilled water. The reaction mixture in a sealed conical flask was incubated at 50 °C in the dark for 7 days. After incubation, the hydroperoxide from linoleic acid was determined using the thiocyanate method (Mitsuda et al., 1966). An aliquot (200 μL) of the reaction mixture was mixed with 75% ethanol (9.4 mL), 30% ammonium thiocyanate (200 μL), and 20 mM $FeCl_2$ (200 μL), and the absorbance of this colored solution was measured at 500 nm. A control containing no added sample represents 100% lipid peroxidation. Antioxidants at 20 and 100 μM levels were used for this experiment. Toc was used as the standard sample.

Antioxidative Assay of Rabbit Erythrocyte Membrane Ghost System. Commercially available rabbit blood (100 mL) was obtained from Japan Biotest Institute Co., Ltd., and diluted with 100 mL of isotonic buffer solution (10 mM phosphate/152 mM KCl, pH 7.4). The diluted blood solution was centrifuged at 1500*g* for 20 min, and the red blood cells were collected, washed three times with 100 mL of the isotonic buffer solution, and lysed in 10 mM phosphate buffer (pH 7.4). Erythrocyte membrane ghosts were pelleted by centrifugation (20000*g*, 40 mg of protein/mL) (Osawa et al., 1987). Peroxidation of the erythrocyte membrane ghosts induced by *tert*-butyl hydroperoxide (*t*-BH) was carried out according to the method of Ames et al. (1981). Eriocitrin metabolites and Toc were dissolved in dimethyl sulfoxide, and the sample solution (100 μL) was mixed with the ghost suspension (850 μL) and 24 mM *t*-BH solution (50 μL). After incubation at 37 °C for 20 min, 1 mL of 2.0 M TCA/1.7 M HCl and 2 mL of 0.67% thiobarbituric acid (TBA) solution were added to stop the reaction. The quantity of TBA reactive substance (TBARS) was determined at 532 nm. A control containing no added sample represents 100% lipid peroxidation. Antioxidants were used for this experiment. Toc was used as the standard sample.

RESULTS AND DISCUSSION

Analysis of Eriocitrin Metabolites by Intestinal Bacteria. The metabolism of eriocitrin was examined using 18 different intestinal bacteria. Intestinal bacteria were cultured in their respective medium containing eriocitrin or eriodictyol. Eriocitrin was changed by the bacteria (*Bacteroides*, *Bifidobacterium*, *Propionibacterium*, *Enterobacter*, *Enterococcus*, *Lactobacillus*, and *Streptococcus*), except for *Clostridium.* Especially, *Ba. uniformis* JCM 5828, *Ba. distasonis* JCM 5825, and *Enterobacter cloacae* IAM 12349 changed eriocitrin by >25%. Eriocitrin appeared to be metabolized by these bacteria. On the other hand, eriodictyol was changed >25% only by *C. butyricum* IFO 14252; the others caused no change. This indicated that eriodictyol was metabolized by *C. butyricum* IFO 14252 and that the bacteria had the ability to cleave the flavonoid ring of eriodictyol.

We attempted to identify eriocitrin metabolites by culturing the intestinal bacteria. *Ba. distasonis* JCM 5825 was cultured with the GAM semisolid medium containing eriocitrin for 3 days. According to HPLC analysis (data not shown), after culture, the peak of eriocitrin decreased and a novel peak appeared. The novel peak was estimated to be that of eriocitrin metabolites. The compound of the peak was isolated using preparative HPLC, and the isolated compound was analyzed with HPLC, ^{1}H NMR, and ^{13}C NMR. The

compound exhibited agreement with the retention time for an authentic sample of eriodictyol in HPLC analysis. ^{1}H-NMR data (in CD_3OD-*d*) exhibited δ 5.21 (1H, dd, J = 13 and 3 Hz, H2), 3.00 (1H, dd, J = 17 and 13 Hz, H3a), 2.64 (1H, dd, J = 17 and 3 Hz, H3b), 5.85 (1H, d, J = 2 Hz, H6), 5.83 (1H, d, J = 2 Hz, H8), 6.86 (1H, s, H2′), 6.73 (1H, d, J = 1 Hz, H5′), 6.73 (1H, d, J = 1 Hz, H6′), and ^{13}C-NMR spectra (in CD_3OD-*d*) exhibited δ 44.3, 80.7, 96.5, 97.3, 103.6, 115.0, 116.5, 119.5, 132.1, 146.7, 147.1, 165.1, 165.7, 168.6, 198.0. The compound was identified as eriodictyol, which was confirmed by direct comparison with an authentic sample in ^{1}H NMR and ^{13}C NMR. We confirmed that eriocitrin was changed to eriodictyol by culturing the other bacteria, except the *Clostridium* species, for HPLC analysis (data not shown). The β-glycoside bond of eriocitrin was suggested to be cleaved by these bacteria, and eriodictyol of the aglycon appeared.

On the other hand, *C. butyricum* IFO 14252 was cultured for 2 days with TGC medium containing eriodictyol. After incubation, HPLC analysis was carried out (data not shown); the peak of eriodictyol was found to decrease and a novel peak appeared. The novel peak was estimated to be the degraded products of eriodictyol. The material of the novel peak was purified using preparative HPLC. The purified material was identified as 3,4-DHCA from the agreement with the spectral peak in GC/MS analysis of an authentic sample. We also confirmed that the peak was identified as 3,4-DHCA from the results, in which the retention time of the peak agreed with that of 3,4-DHCA as a standard reagent for HPLC analysis. The C ring of eriodictyol was suggested to be cleaved by *C. butyricum,* and 3,4-DHCA appeared.

Pathway of Eriocitrin Metabolized by Intestinal Bacteria. The fecal bacteria had been shown to hydrolyze flavonoid glycosides to the corresponding aglycons (MacDonald et al., 1983). Three obligate anaerobic bacteria isolated from the human intestinal flora had also been reported capable of hydrolyzing glycoside to aglycons: *Ba. distasonis* hydrolyzed robinin to kaempferol and *Ba. uniformis* and *Ba. ovatus* converted rutin to quercetin (Morris et al., 1985). The isolation and tentative identification of human intestinal bacteria that cleave the C ring of flavonoids were attempted. *Clostridium* strains isolated from the human fecal flora were capable of cleaving the C ring of quercetin, kaempferol, and naringenin at C3−C4. Mixed cultures of a flavonoid anaerobic environment rapidly metabolized rutin to 3,4-dihydroxyphenylacetic acid and PHL (Winter et al., 1989; Krishnamurty et al., 1970).

In this study, we showed that eriocitrin of the antioxidants in lemon fruit was metabolized to eriodictyol, 3,4-DHCA, and PHL. We estimated the metabolic pathways of eriocitrin by culturing intestinal bacteria as shown in Figure 1. Eriocitrin was dehydrated to eriodictyol of its aglycon by culturing *Ba. distasonis.* Eriodictyol was degraded to 3,4-DHCA with its C-ring cleavage by culturing *C. butyricum.* We believed from the conformation of eriodictyol that PHL appeared at the same time. However, PHL was not detected. As for quercetin, it had been shown to be converted to 3,4-dihydroxyphenylacetic acid and PHL by a *Butyrivibrio* species from ruminal fluid (Krishnamurty et al., 1970). However, it had been reported that PHL was not detected in metabolites of quercetin using human intestinal bacteria and might be degraded further

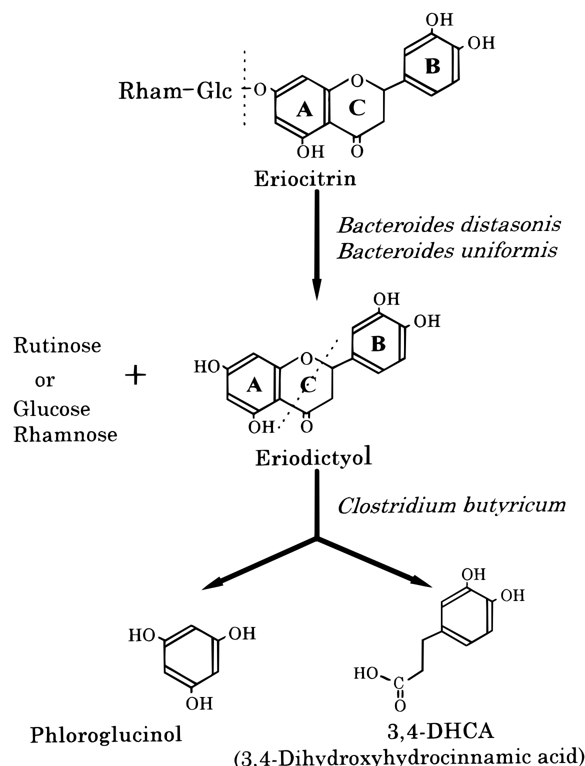

Figure 1. Metabolic pathways of eriocitrin by intestinal bacteria.

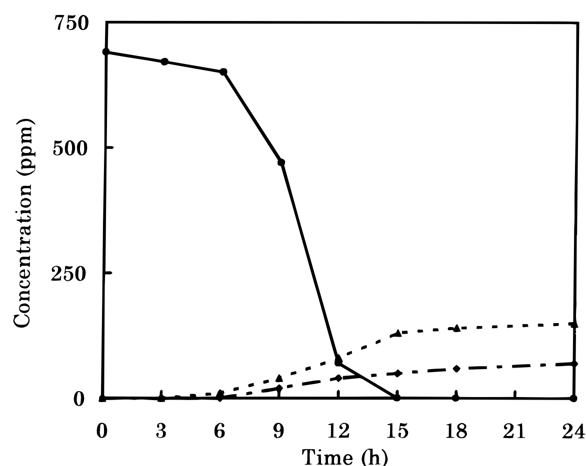

Figure 2. Time course for metabolites of eriocitrin by culturing of human fecal bacteria. Eriocitrin (●), eriodictyol (▲), and 3,4-DHCA (◆) were determined by HPLC analysis; details are given under Materials and Methods. Values in the figure are the average of duplicates.

(Winter et al., 1989). We also thought that PHL was not detected because it was further metabolized by the bacteria.

Metabolism of Eriocitrin by Human Fecal Bacteria. Human feces have been known to contain abundant intestinal bacteria. To propose the human metabolism pathway of eriocitrin, we examined the metabolites of eriocitrin using human fecal bacteria as shown in Figure 2. The bacteria from human feces were cultured with BHI medium containing eriocitrin. The eriocitrin and its metabolites (eriodictyol and 3,4-DHCA) were determined by HPLC analysis. As shown in Figure 2, eriocitrin decreased along with the culturing of human fecal bacteria and disappeared after 15 h of culturing. Eriodictyol then appeared after 6 h of culturing, and 3,4-DHCA appeared after 9 h of culturing; both

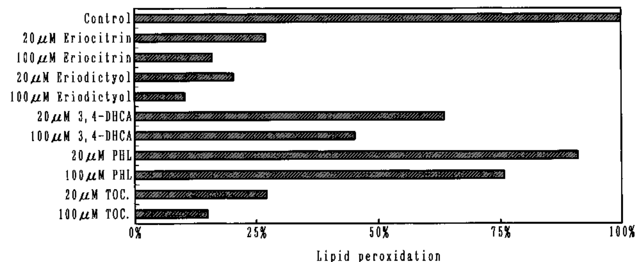

Figure 3. Antioxidative activity of eriocitrin, eriodictyol, 3,4-DHCA, PHL, and Toc in the linoleic acid system measured by the thiocyanate method. A control containing no added samples represents 100% lipid peroxidation. Antioxidants at 20 and 100 μM were used for this experiment; details are given under Materials and Methods. Values in the figure are the average of duplicates.

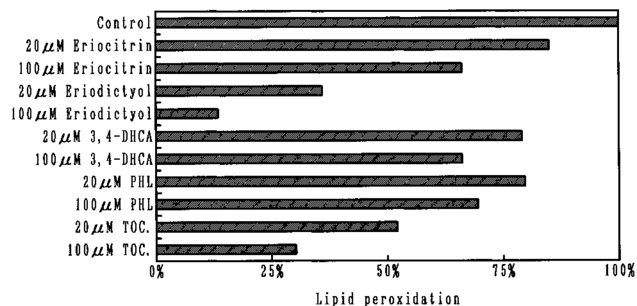

Figure 4. Antioxidative activity of eriocitrin, eriodictyol, 3,4-DHCA, PHL, and Toc in the rabbit erythrocyte membrane ghost system. Antioxidants at 20 and 100 μM were used for this experiment; details are given under Materials and Methods. Values in the figure are the average of duplicates.

metabolites increased along with culturing. Eriocitrin was indicated to be metabolized by human intestinal bacteria as shown in Figure 1. This indicates that the antioxidative eriocitrin from lemon fruit is metabolized *in vivo*. We thought that eriocitrin was not completely metabolized to 3,4-DHCA and PHL *in vivo* but that it was metabolized partially and that each of the eriocitrin metabolites was absorbed within the body *in vivo*. We plan to explore this point in the future.

Antioxidative Activity of Eriocitrin Metabolites. We had reported that the antioxidative activity of eriocitrin in the linoleic acid system was similar to that of Toc (Miyake et al., 1997). The antioxidative activity of eriocitrin metabolites was examined using the linoleic acid system as shown in Figure 3. Eriodictyol was shown to have higher activity than eriocitrin or Toc, but the activity of 3,4-DHCA and PHL was lower. However, this showed that the metabolites of eriocitrin preserved the antioxidative activity.

Like many other biological membranes, red blood cell membranes are prone to lipid peroxidation because of their high polyunsaturated lipid content. Therefore, the evaluation of the antioxidative activity of eriocitrin metabolites was performed in the rabbit erythrocyte membrane system as shown in Figure 4. The results are the same as those with the linoleic acid system. Eriodictyol had a higher activity than Toc. 3,4-DHCA and PHL exhibited only weak activity, but the metabolites of eriocitrin preserved the antioxidative activity. Like the results with the liposome system, the affinity of eriodictyol for the erythrocyte membrane caused a more effective inhibition of malondialdehyde (MDA) formation than eriocitrin, 3,4-DHCA, and PHL. We speculated that eriodictyol appeared to be an effective antioxidant *in vivo* when eriodictyol of eriocitrin metabolites was absorbed within the body.

It had been reported that many flavonoid and phenolic compounds such as hesperidin and naringin were abundant in citrus fruits (Albach and Redman, 1969). Table 1 shows the antioxidative activity of the flavonoid glycosides and their aglycons using the rabbit erythrocyte membrane model of autoxidation. The aglycons of flavonoid glycosides were shown to exhibit stronger activity than the flavonoid glycosides. The aglycon of flavonoid glycosides containing adjacent dihydroxy groups on the B ring such as eriodictyol and quercetin showed high antioxidative activity. Eriodictyol, one of the metabolites of eriocitrin, exhibited strong activity among these flavonoid compounds.

Table 1. Antioxidative Activity of Flavonoid Glycosides of Citrus Fruit and Their Aglycons in the Rabbit Erythrocyte Membrane Ghost System[a]

antioxidant	concn (μM)	activity (%)	antioxidant	concn (μM)	activity (%)
eriocitrin	50	85	rutin	50	49
	100	66		100	35
eriodictyol 7-glucoside	50	79	quercetin	50	42
	100	64		100	16
eriodictyol	50	36	diosmin	50	102
	100	14		100	88
hesperidin	50	84	diosmetin	50	60
	100	65		100	45
hesperetin	50	63	Toc	50	52
	100	50		100	30
naringin	50	91			
	100	86			
naringenin	50	80			
	100	73	control		100

[a] A control containing no added samples represents 100% lipid peroxidation. Antioxidants at 50 and 100 μM were used for this experiment; details are given under Materials and Methods. Values are the average of duplicates.

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