JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 2009−2014 **2009**

Antioxidant Activity of Citrus Limonoids, Flavonoids, and Coumarins

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A variety of in vitro models such as β-carotene−linoleic acid, 1,1-diphenyl-2-picryl hydrazyl (DPPH), superoxide, and hamster low-density lipoprotein (LDL) were used to measure the antioxidant activity of 11 citrus bioactive compounds. The compounds tested included two limonoids, limonin (Lim) and limonin 17-β-D-glucopyranoside (LG); eight flavonoids, apigenin (Api), scutellarein (Scu), kaempferol (Kae), rutin trihydrate (Rut), neohesperidin (Neh), neoeriocitrin (Nee), naringenin (Ngn), and naringin-(Ng); and a coumarin (bergapten). The above compounds were tested at concentration of 10 μM in all four methods. It was found that Lim, LG, and Ber inhibited <7%, whereas Scu, Kae, and Rut inhibited 51.3%, 47.0%, and 44.4%, respectively, using the β-carotene−linoleate model system. Lim, LG, Rut, Scu, Nee, and Kae showed 0.5% 0.25%, 32.2%, 18.3%, 17.2%, and 12.2%, respectively, free radical scavenging activity using the DPPH method. In the superoxide model, Lim, LG, and Ber inhibited the production of superoxide radicals by 2.5−10%, while the flavonoids such as Rut, Scu, Nee, and Neh inhibited superoxide formation by 64.1%, 52.1%, 48.3%, and 37.7%, respectively. However, LG did not inhibit LDL oxidation in the hamster LDL model. But, Lim and Ber offered some protection against LDL oxidation, increasing lag time to 345 min (3-fold) and 160 min (33% increase), respectively, while both Rut and Nee increased lag time to 2800 min (23-fold). Scu and Kae increased lag time to 2140 min (18-fold) and 1879 min (15.7-fold), respectively. In general, it seems that flavonoids, which contain a chromanol ring system, had stronger antioxidant activity as compared to limonoids and bergapten, which lack the hydroxy groups. The present study confirmed that several structural features were linked to the strong antioxidant activity of flavonoids. This is the first report on the antioxidant activity of limonin, limonin glucoside, and neoeriocitrin.

KEYWORDS: Antioxidant activity; limonoids; flavonoids; coumarins, citrus paradisi

INTRODUCTION

Diets high in fruits and vegetables are protective against a variety of diseases, particularly cardiovascular diseases and some types of cancer (*1*). Reactive oxygen, (e.g., superoxide and singlet oxygen radicals) and nitrogen (e.g., peroxynitrite and nitrogen dioxide radical species generated in vivo) are known to alter cellular structure and function. These induced alterations are thought to cause chronic degenerative diseases including heart disease and cancer (*2*). Antioxidant bioactive compounds are a class of nutrients that has been shown to reduce the incidence of these diseases (*3*).

Citrus fruits are rich sources of vitamin C (ascorbic acid), an essential nutrient with well-described antioxidant properties. However, recent studies have demonstrated that citrus also contain other bioactive compounds including flavonoids, coumarins, carotenoids, and limonoids with potential health-promoting properties (*4*−*8*). Accumulative evidence suggest antioxidant activities of flavonoids from a variety of plant sources (*9*−*13*). Indeed, flavonoids possess a wide range of activities in vitro (*14*, *15*). For example, this class of bioactive compounds is known to act as free radical scavengers, to modulate enzymatic activities, and to inhibit cellular proliferation as well as possessing antibiotic, anti-allergenic, anti-diarrhea, anti-ulcer, and anti-inflammatory activities (*16*). Flavonoids were shown to scavenge peroxyl radicals, alkyl peroxyl radicals, superoxide hydroxyl radicals, and peroxynitrite in aqueous and organic environments (*8*−*12*, *16*).

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10.1021/jf0484632 CCC: $30.25
Published on Web 03/01/2005

Coumarins, derived from a branch of the phenylalanine metabolism pathway that leads ultimately to furanocoumarin (psoralin) synthesis are another class of bioactive compounds found in citrus. Some coumarins have been shown to possess anticarcinogenic and antithrombotic activities (*17*−*20*). Limited data are available to demonstrate the antioxidant activities of coumarins (*12*, *20*, *21*).

Citrus limonoids are a group of highly oxygenated triterpenoids present mainly in the Rutaceae and Meliaceae families. Research with these compounds has shown that some limonoids could induce the detoxifying enzyme glutathione *S*-transferase in the liver of mice and rats (*4*). Citrus limonoids were also shown to inhibit the formation of chemically induced neoplasia in the oral cavity, forestomach, small intestine, colon, lung, and skin of laboratory animals (*5*, *6*). Our previous studies (*7*) and elsewhere (*8*) have also shown that limonoids can inhibit the proliferation of breast cancer cells grown in culture. However, the antioxidant activities of citrus limonoids have not yet been well-documented.

The objective of this study was to determine the antioxidant activity of grapefruit (*Citrus paradisi* Macf.) bioactive compounds by using four in vitro models. The compounds selected included two limonoids, limonin (Lim) and limonin 17-β-D-glucopyranoside (LG); eight flavonoids, apigenin (Api), scutellarein (Scu), kaempferol (Kae), rutin trihydrate (Rut), neohesperidin (Neh), neoeriocitrin (Nee), naringenin (Ngn), and naringin (Ng); and a coumarin bergapten (Ber).

MATERIALS AND METHODS

Materials. Limonin (Lim, >98% pure) and limonin 17-β-D-glucopyranoside (LG) (90% pure) were purified according to the established procedures in our lab (*7*, *22*) from grapefruit seeds at the Texas A&M University—Kingsville Citrus Center, Weslaco, TX. Other citrus compounds: rutin trihydrate (Rut), apigenin (Api), scutellarein (Scu), kaempferol (Kae), neohesperidin (Neh), neoeriocitrin (Nee), naringenin (Ngn), naringin (Ng), and bergapten (Ber) were purchased (Indofine Chemical Company, Somerville, NJ). Other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Spectra measurements were obtained using a DU 640 UV−Vis spectrophotometer (Beckman Coulter, USA).

Isolation of Limonoids. Seeds of mature grapefruits were collected and dried at 55 °C. Approximately, 500 g of the dried seeds was ground with a Retch mill (Brinkmann, Westbury, NY), and it was extracted with hexane using a Soxhlet extractor for overnight to remove the oil. Then, the spent was sequentially extracted using acetone and methanol for 10 h separately. Both the fractions were filtered and concentrated using a rotary evaporator under vacuum (<60 °C). Acetone concentrate was partitioned with methylene chloride−water (1:1) using an ultrasonic sonicator for 2−3 times. The methylene chloride fractions were separated, pooled, concentrated, and kept free of limonin crystallization. Limonin crystals were obtained by filtration under vacuum. The aqueous layer was mixed with methanol extract and concentrated under vacuum and loaded to the XAD-2 column. The column was first washed thoroughly with water, and limonoid glucosides were eluted with methanol. The methanol fraction was concentrated under vacuum and subjected to preparative HPLC.

Preparative HPLC. Limonoids were separated using Waters prep HPLC system (Waters Corporation, Milford, MA). Mobile phase used was 10% acetonitrile in 0.03 mM phosphoric acid (solvent A) and 24% acetonitrile in 0.03 mM phosphoric acid (solvent B). The linear gradient program was 0 min − 100% A; 120 min − 0% A with a flow rate was set 25 mL/min, and detection was carried out at 210 nm. A total of 200 μL of methanol extract was injected to HPLC, and different fractions were collected and, as per the peaks retention time and collected fractions, were analyzed by analytical HPLC for their purity. Fractions having similar retention time were pooled, concentrated under vacuum, and freeze-dried to obtain limonoid glucoside.

HPLC Analysis. The HPLC system consisted of a Thermo Electron Corporation P-400 quaternary HPLC pump (Thermo Electron Corporation, USA), Membrane degasser LDC analytical and Spectra system AS3000 autosampler (Thermo Separation Products). Peaks were analyzed with Thermo Separation Products PDA detector. Chromatographic separations were accomplished on Chemcisorb-5-ODS column (150 × 6.0 mm, 5 μm particle size) (ChemcoPak, Osak, Japan). Elution was carried out at room temperature under gradient conditions with a mobile phase consisting of 10% acetonitrile in 0.03 mM phosphoric acid (solvent A) and 24% acetonitrile in 0.03 mM phosphoric acid (solvent B), linear gradient program was 0 min − 100% A; 40 min − 0% A with a flow rate was 1.0 mL/min, and detection was carried out at 210 nm. All standards and samples were filtered through a 0.45-μm Millipore filter and injected (50 μL) to HPLC. The compounds were quantified using Chemquest software. Finally, the isolated compounds were identified using EI-MS as described earlier from our lab (*22*).

Antioxidant Activity by β-Carotene−Linoleic Acid Bleaching Assay. This experiment was carried out by the method of Emmons et al. (*23*) and Chen and Ho (*24*). Emulsion was prepared by dissolving β-carotene (5 mg) in 50 mL of chloroform, and 3 mL was added to the mixture of linoleic acid (40 mg) and Tween 40 (400 mg). Chloroform was removed under a stream of nitrogen gas, and oxygenated water (100 mL) was added to the emulsion and vigorous mixing with a vortex-type mixer. Aliquots (3 mL) of the β-carotene−linoleic acid emulsion were mixed with 40 μL of sample solution (10 μM) and incubated in a water bath at 50 °C. Oxidation of the emulsion was monitored with a spectrophotometer by measuring absorbance at 470 nm over a 60-min period. The negative control contained 40 μL of ethanol in place of the compounds. The antioxidant activity is expressed as percent inhibition relative to the negative control after 60 min incubation using the following equation:

$$AA = 100(DR_C - DR_S)/DR_C$$

where AA is the antioxidant activity, DR_C is the degradation rate of the control [=(ln(a/b))/60], DR_S is the degradation rate in the presence of the sample [=(ln(a/b))/60], a is the initial absorbance at 0 time, and b is the absorbance at 60 min. All the citrus compounds were evaluated at the final concentration of 10 μM, and Rut served as the positive control.

DPPH Radical Scavenging Activity. Samples were tested individually at a final concentration of 10 μM by addition to an ethanolic solution of 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical (100 μM). The mixtures were vigorously mixed and allowed to stand in the dark for 30 min at 25 °C. The absorbance of the resulting solution was measured using a spectrophotometer at 517 nm against a blank sample without DPPH, the negative control (*24*−*26*), and Rut served as the positive control.

Superoxide Radical Scavenging Activity. The superoxide radical scavenging effect of the citrus bioactive compounds was determined by monitoring the reduction of nitro blue tetrazolium (NBT) (*27*). The reaction mixture contained the test compound (10 μM) in combination with PMS (5-methylphenazinium methosulfate) (20 μM), NADH [adenosine 5′-(trihydrogen diphosphate)] (156 μM), and NBT (50 μM) in phosphate buffer (0.1 M, pH7.4) in a final volume of 2.5 mL (*27*). The samples were incubated at ambient temperature for 5 min and reaction product formation measured at 560 nm using a spectrophotometer. The blank sample (the negative control) was run without NBT, and Rut served as the positive control.

Hamster Low-Density Lipoprotein (LDL) Oxidation by Conjugated Diene Formation. Male Syrian Golden Hamsters (SASCO-strain, Charles River Laboratories, Wilmington, MA) fed with a casein-based diet (*28*) for 7 weeks were used as plasma donors. Plasma was harvested from cardiac blood, drawn into tubes containing sufficient EDTA to inhibit blood clotting. Following centrifugation at 2500*g* for 20 min at 4 °C, prepared plasma was stored at 4 °C prior to LDL isolation and preparation for the conjugated diene formation assay (*29*). Conjugated diene formation was used as an endpoint, following initiation of oxidation by the addition of $CuSO_4$ (10 μM) to the mixture of individual citrus compounds (10 μM) and LDL (20 μg of protein/ml) in 0.1 M phosphate-buffered saline (PBS) (pH = 7.4, in 0.15 M NaCl). Copper-

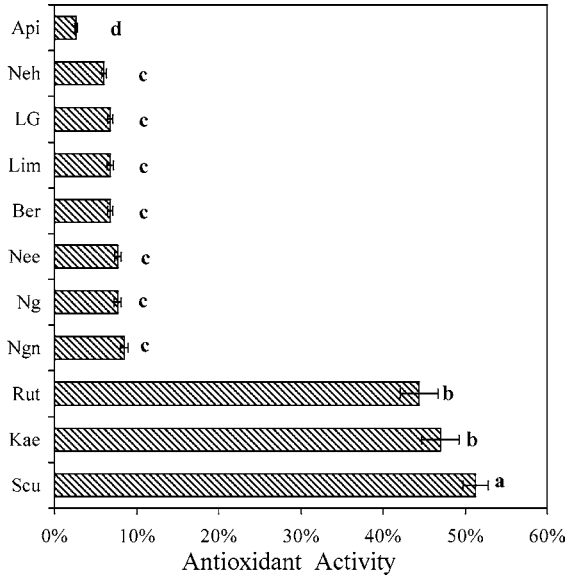

Figure 1. Antioxidant activity of limonoids, flavonoids, and a coumarin in a β-carotene–linoleic acid bleaching assay system. All the compounds tested were at the final concentration of 10 μM. The values represent the autoxidation of the linoleic acid/β-carotene emulsion expressed as percentage inhibition of auto-oxidation observed in the negative control lacking citrus compounds; Rut served as the positive control. Values are means ± SEM (n = 3). Values with different letters are significantly different at $P \leq 0.05$.

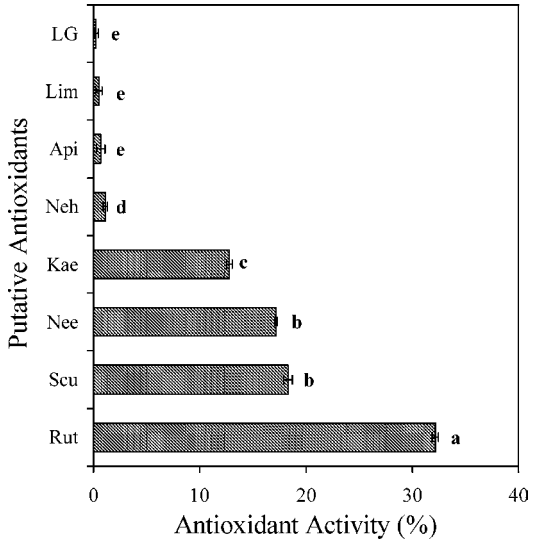

Figure 2. Ability of citrus limonoids, flavonoids, and a coumarin to scavenge DPPH radicals. All the compounds tested were at the final concentration of 10 μM. The values represent the percentage of DPPH reduction observed in negative control incubations lacking citrus compounds; Rut served as the positive control. Values are means ± SEM (n = 3). Values with different letters are significantly different at $P \leq 0.05$. Bergapten, Ng, and Ngn demonstrated negative activity.

catalyzed oxidation of LDL was monitored by continuous measurement of absorbance at 234 nm with a spectrophotometer at 37 °C. Lag time was calculated as the intersection of baseline slope with that of the slope during propagation of conjugated diene formation. The blank sample (the negative control) was run without compounds and Rut served as the positive control.

Statistical Analysis. All tests were run in triplicate, and values were expressed as means ± SEM. Differences among treatments were evaluated by one-way ANOVA (SAS) with post hoc means ranking test using Duncan. Mean values differing by $P \leq 0.05$ were considered as significant.

RESULTS

The antioxidant activity of two citrus limonoids, eight flavonoids, and a courmarin bergapten as measured by the bleaching of β-carotene are presented in **Figure 1**. At the final concentration of 10 μM, Lim, LG, and Ber inhibited <7% of the carotene bleaching observed in the negative control incubations. Flavonoids exhibited a much greater antioxidant activity, with Scu and Kae inhibiting carotene bleaching by 51.3% and 47.0%, respectively, stronger than the positive control Rut, 44.4%; however, the other compounds tested showed weaker antioxidant activity than Rut.

The DPPH free radical scavenging potentials of the 11 citrus bioactive compounds at the concentration of 10 μM are given in **Figure 2**. Flavonoids showed much stronger DPPH radical scavenging activities than limonoids. Rutin as the positive control showed the highest activity (32.18%), followed by Scu (18.32%), Nee (17.18%), and Kae (12.79%). With Lim and LG, the free radical scavenging activities were 0.5% and 0.25%, respectively. Naringin, Ngn, and Ber demonstrated negative activity; however, Ng, Ngn, and Ber showed 16.5%, 17.3%, and 12.6% scavenging activity respectively at the concentration of 20 μM (data not presented).

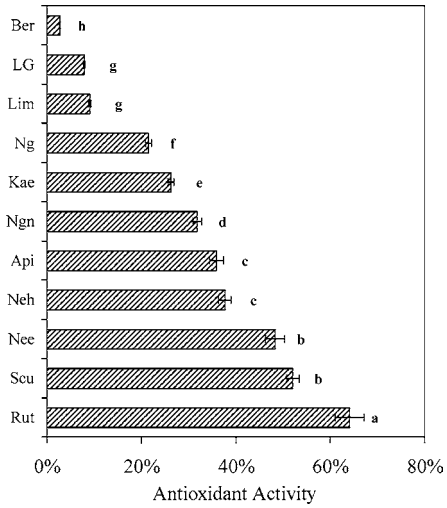

Figure 3. Ability of limonoids, flavonoids, and a coumarin to inhibit NBT (nitro blue tetrazolium) reduction. All the compounds tested were at the final concentration of 10 μM. The values represent the percent inhibition of NBT reduction observed in negative control incubations lacking citrus compounds; Rut served as the positive control. Values are means ± SEM (n = 3). Values with different letters are significantly different at $P \leq 0.05$.

Figure 3 illustrates the ability of the 11 citrus bioactive compounds to scavenge superoxide radicals, and these compounds at the final concentration of 10 μM variably diminished the in vitro superoxide radicals production. The limonoids and bergapten inhibited the production of superoxide radicals by 2.5−10% as compared to the negative control incubations. In contrast, flavonoids inhibited superoxide radicals formation by 22−64%. Among the flavonoids, Rut (the positive control), Scu, Nee, and Neh inhibited superoxide formation by 64.08%, 52.06%, 48.3%, and 37.7%, respectively.

The ability of the 11 citrus bioactive compounds to prevent copper-initiated accumulation of conjugated diene fatty acid oxidation products in hamster LDL is depicted in **Table 1**. The

Table 1. Citrus Bioactive Compounds Effect on Initiation Time for Copper-Mediated Conjugated Diene Formation in Hamster LDL

compound[a]	initiation time (min)[b]
Rut	2800 ± 20.3 a[c]
Nee	2800 ± 22.4 a
Scu	2140 ± 15.6 b
Kae	1879 ± 14.8 c
Neh	400 ± 5.1 d
Lim	345 ± 4.8 e
Api	340 ± 5.1 e
Ber	160 ± 4.3 f
Ngn	150 ± 4.2 f
Ng	150 ± 5.1 f
LG	120 ± 3.5 g
control	120 ± 3.2 g

[a] The concentration of compounds was 10 μM. [b] Each value is the mean ± SEM, $n = 3$. [c] Values with different letters are significantly different at $P \leq 0.05$.

average initiation time for LDL incubated in the absence of citrus bioactive compounds (the negative control) was 120 min. As shown in **Table 1**, 10 μM LG did not inhibit LDL oxidation; Lim and Ber, however, did offer some protection against LDL oxidation, increasing lag time to 345 min (3-fold) and 160 min (33% increase), respectively. Compared to the limonoids, flavonoids showed much stronger protection against LDL oxidation. This was especially true for Rut (the positive control) and Nee, where lag time increased 23-fold to 2800 min, while Scu and Kae increased lag time to 18-fold (2140 min) and 15.7-fold (1879 min), respectively.

DISCUSSION

The present data demonstrated that each antioxidant differed in its antioxidative capacity toward different sources of free radicals and other oxidants. The antioxidant activities of the 11 citrus bioactive compounds were tested with four different model systems. The β-carotene−linoleic acid bleaching method relies on oxygen mediated linoleic acid free radical formation; the DPPH method is based on DPPH free radical mediated oxidation; while the superoxide method relies on oxidation induced by superoxide and its decomposition products. The LDL method assesses lipid oxidation initiated by copper-mediated Fenton chemistry. Potential bioactive compounds using multiple in vitro assay systems are considered advantageous to assess the antioxidant properties of food components as different reaction mechanisms can be evaluated.

A comparison of the activity of the 11 bioactive compounds in the four assays provides some insight on potential mechanisms of action. For example, Nee demonstrated strong antioxidant activity in the hamster LDL system and weak activity in the β-carotene−linoleic acid system. It is possible that Nee not only scavenged the radicals (offered hydrogen or electrons) but also chelated the Cu^{2+} in the LDL assay inhibiting Fenton chemistry-mediated oxidation. However, it appears that Nee only released limited hydrogen or electrons to the system and demonstrated the weak antioxidant activity based on β-carotene−linoleic acid method.

The data presented in this experiment indicated that the marked antioxidant activity of some flavonoids seemed to be due to the fact that the active chemicals are polyphenol compounds containing a chromanol ring system with the capacity to stabilize unpaired electrons and thereby scavenge free radicals.

Pietta (*30*) and Bors et al. (*31*) suggested that the radical-scavenging activity of flavonoids depended on the structure and substituents of the heterocyclic rings and the B ring. The major determinants for radical-scavenging capability were found to be (a) the presence of a catechol group in ring B, which has better electron-donating properties and is a radical target, and (b) a 2,3-double bond conjugated with the 4-oxo group, which is responsible for electron delocalization. The presence of a 3-hydroxy group in the heterocyclic ring also increases the radical-scavenging activity, while additional hydroxyl or methoxyl groups at positions 3, 5, and 7 of rings A and C seem to be less important (*30*, *31*). In addition to the location and total number of hydroxyl groups, the solubility of the phenolics in the test medium may significantly affect their ability to act as antioxidants (*11*). For example, antioxidant activity of flavonoids in lard appears to be related to the number of *O*-dihydroxy grouping in the A and B rings (*32*), whereas a lack of conjugation between the B and C rings is a major influence in aqueous media (*33*).

The results from the present studies indicated that the hydroxyl group in position 6 of ring A (as in scutellarein) could increase the antioxidant activity of flavonoids. These structural features contribute to increase the antioxidant capability of parent flavonoids. Thus, flavonols and flavones containing a catechol group in ring B are highly active, with flavonols more potent than the corresponding flavones because of the presence of the 3-hydroxyl group. Glycosylation of this group, as in Rut, reduces the radical-scavenging capacity. Methylation of the catechol moiety hydroxyl and the presence of only one hydroxyl in ring B diminish the activity as in Neh, Api, Kae, and Scu. Flavanones, such as in Ngn and Ng, due to the lack of conjugation provided by the 2,3-double bond with the 4-oxo group, are weak antioxidants.

Limonoids are highly oxygenated triterpenoids with fewer hydroxyl groups than flavonoids. Both of these structural features probably contribute to the weak antioxidant activity seen with these compounds. However, the poor aqueous solubility of Lim may have limited its antioxidation capacity in the present study. Our data demonstrated that Lim had relatively stronger antioxidant activity than LG, especially in the LDL oxidation assay system. Glycosylation is known to limit the antioxidant activity of the flavonoids kaempferol (*34*) and quercetin (*13*) in vitro.

Previous studies have shown inconsistent antioxidant activity for Api, Kae, Rut, Neh, Ngn, and Ng using different antioxidant assays (*13*, *35*). Kaempferol has been reported to have stronger antioxidation capacity than Api, and Api was stronger than Rut in the comparison of the total antioxidant activities of flavonoids in human lymphocytes using the comet assay (*13*). In a human LDL oxidation assay, Kae showed weaker antioxidant activity than Rut (*35*). In addition, the stoichiometry of the reaction of the flavonoids with the galvinoxyl free radical using electron spin resonance (ESR) spectroscopy showed the antioxidant potential as Rut > Kae > Api; however, the kinetic measurements demonstrated the antioxidant potential as Kae > Rut > Api, where Api showed little activity as compared with Rut (*36*). In addition, Rut has often served as a positive standard for DPPH and other radical-scavenging assays (*37*−*39*), where it demonstrated good antioxidation capacity as shown in the present study. In our current study, Rut in LDL oxidation assay increased the lag phase about 23-fold, and Kae increased it about 18-fold as compared to the negative control, supporting the literature that Rut is more effective than Kae against lipid peroxidation. In the thiobarbituric acid assay using rat brain and

kidney homogenates, Ng and Ber have also been reported to inhibit lipid peroxidation slightly at 10.09% and 22.15%, respectively (*12*). Naringin and Ngn were reported consistently as having little antioxidant effect in lipid peroxidation systems (*40*, *41*); however, Ng and Ngn were reported to perform inconsistently in the DPPH assay (*42*, *43*). It has also been reported that Scu is more potent than kaempferol-3-*O*-gal in inhibiting lipid peroxidation dependent on Fe^{3+}−ADP/NADPH (*9*). Neohesperidin has been reported as an alkylperoxyl radical scavenger (*44*). In the present study, both Ng and Ngn demonstrated little antioxidant activity in the lipid peroxidation system and in the DPPH assay with inhibition of oxidation less than 25%. Bergapten demonstrated mild antioxidant activity ranging from 0 to 30% as compared to the controls in the different assays. However, Ng, Ngn, and Ber all showed less than 20% antioxidant activity in the DPPH assay at the concentration of 20 μM. Considerable variation of antioxidant activity of Api was observed ranging from 0- to 3-fold compared to the negative controls. The present data with these flavonoids are again consistent with previous publications.

Conclusions. To the best of our knowledge this is the first study on the antioxidant activity of citrus limonoids. In all of the in vitro assays, Lim and LG were very weak antioxidants. However, as has been observed for flavonoids, the limonin aglycone possessed a relatively stronger antioxidant capacity than the limonin glucoside, especially in metal-initiated lipid oxidation. This is the first report on the antioxidant activity of neoeriocitrin (Nee) within metal-initiated lipid oxidation, superoxide radical and DPPH radical-scavenging assays. This citrus compound proved an effective antioxidant in each of these assays, ranking 2nd, 3rd and 3rd strongest, respectively of the 11 compounds tested in each assay system. The data further indicated that the hydroxyl group in position 6 of ring A (as in scutellarein versus naringenin) could increase the antioxidant activity of flavonoids. By comparison, the citrus flavonoids demonstrated mild to strong antioxidant activity. The coumarin bergapten provided little to no protection against DPPH or metal-initiated lipid radical mediated oxidation and inhibited less than 10% of the oxidation in the carotene-bleaching and NBT−superoxide assays.

ACKNOWLEDGMENT

The work was supported in part by USDA-CREES Grants 2001-34402-10543 and 2001-52102-11257. We appreciate Dr. John da Graca and Dr. John Jifon for critically reviewing the manuscript. We would like to thank Dr. Qingguo Tian, Shuen-Ei Chen, Kendall Hood, and Jairam Vanamala for their constant help and suggestions.

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Received for review September 13, 2004. Revised manuscript received January 10, 2005. Accepted January 19, 2005.

JF0484632