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Evaluation of the Antioxidant Capacity of Limonin, Nomilin, and Limonin Glucoside

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The antioxidant capacity (AOC) of three representative citrus limonoids, limonin, nomilin, and limonin glucoside, was examined by the oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC), β-carotene−linoleic acid bleaching, and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assays. Pure compounds and proper negative (cinnamic acid) and positive (2,6-di-*tert*-butyl-4-methylphenol (BHT) and ascorbic acid) controls were used to remove any ambiguity in interpreting results. In all cases, limonin and nomilin gave results equivalent to those of cinnamic acid, indicating that they do not possess any inherent AOC and should not be considered antioxidants. Similar results were observed for limonin glucoside, with the exception of an anomalous result obtained from the β-carotene−linoleic acid bleaching assay. Limonin glucoside was deemed not to be an antioxidant on the basis of the three unequivocal assays.

KEYWORDS: Limonin; nomilin; limonin glucoside; citrus; antioxidant capacity.

INTRODUCTION

Citrus fruits and juices are recognized as one of the most healthful components of the human diet. Limonoids are highly oxygenated triterpenoid compounds that occur in high concentrations as aglycones and glucosides (**Figure 1**) in citrus seed and fruit tissues. These compounds have been screened for a number of biological activities, and structural features that influence antitumor (*1*−*7*), anti-HIV (*8*), and cholesterol-lowering properties (*9;10*) have been identified.

Recently, the antioxidant capacity (AOC) of citrus limonoids and limonoid-containing extracts has been evaluated by a number of authors utilizing Racimat experiments (*11*), superoxide radical quenching (*12*), β-carotene−linoleic acid bleaching (*13;14*), and DPPH radical scavenging (*13*) assays. These studies assign AOC to citrus limonoid components ranging from undetectable or limited to equivalent or greater than that of vitamin C.

The broad range of the reported AOC values and the fact that citrus limonoids do not possess the structural features commonly associated with antioxidants (e.g., phenol moiety, an extended conjugated system, electron delocalization potential) invites their reassessment. We now report the AOC evaluation of three pure citrus limonoids (limonin, nomilin, and limonin glucoside) following guidelines established in a series of White Papers from the "First International Congress on Antioxidant Methods" (*15;16*).

MATERIALS AND METHODS

Materials and Chemicals. Water was distilled and deionized. Solvents (Fisher, Pittsburgh, PA) were HPLC grade. 1,1-Diphenyl-2-picryl hydrazyl (DPPH) was purchased from MP Biomedicals (Aurora, OH), and 2,6-di-*tert*-butyl-4-methylphenol (BHT), cinnamic acid, ascorbic acid, 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), β-carotene, Tween 80, linoleic acid, fluorescein disodium salt, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO). Pure crystalline limonin, nomilin, and limonin glucoside were available in our laboratory. All other reagents were analytical grade.

Purity Analysis of Limonoids. The crystalline limonoids (limonin, nomilin, and limonin glucoside) were established as pure on the basis of defined melting points and analytical and spectral data (NMR, HPLC-MS) compared to pure compounds maintained in our laboratory. NMR, HPLC-MS, and direct infusion MS analyses were used to confirm the purity of the limonoids. ^{1}H and ^{13}C NMR spectra were obtained on a Varian-400 (Palo Alto, CA) nuclear magnetic spectrometer operating at 400 and 100 MHz, respectively. Samples were dissolved in CD_3OD, and chemical shifts were reported in ppm downfield from internal tetramethylsilane (TMS). HPLC-MS and MS analysis were conducted as previously described (*17;18*).

DPPH Radical Scavenging Activity Assay. The DPPH radical scavenging activity was assessed in a microtiter-based format on the basis of the methods of Hamburger et al. (*19*) and Bouaziz et al. (*20*) with some modifications. Briefly, in a glass 96-well reaction plate, samples, along with positive (BHT, ascorbic acid) and negative (cinnamic acid) controls (50 μL) prepared in methanol (0.001−1 mg/mL), were combined in triplicate with 155 μM methanolic DPPH (200 μL). Following incubation at room temperature for 30 min, the absorbance at 517 nm was read on a Molecular Devices Spectromax 384-Plus plate reader (Sunnyvale, CA).

ABTS Radical Cation Decolorization Assay (TEAC). Antioxidant capacity as assessed by the ABTS radical cation ($ABTS^{\bullet+}$) decolorization assay was accomplished in a microtiter-based format following the methods of Sellappan et al. (*21*) and Re et al. (*22*) with some

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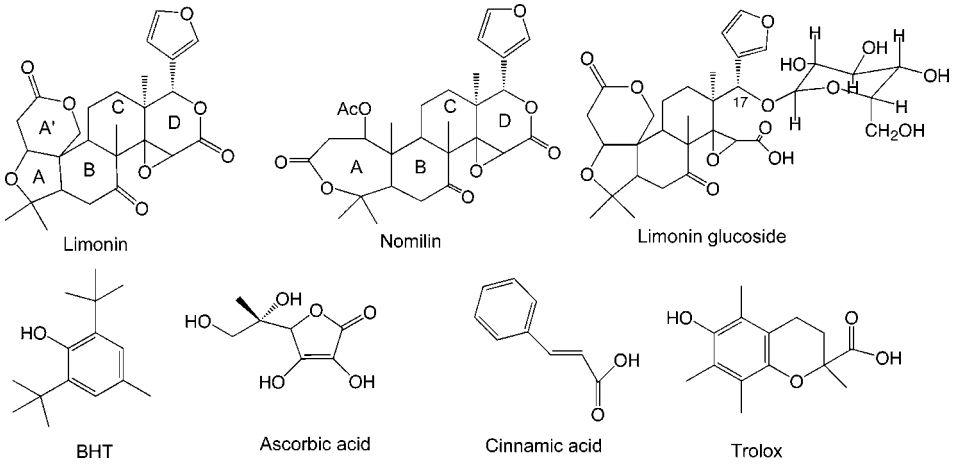

Figure 1. Structures of limonin, nomilin, limonin glucoside, BHT, ascorbic acid, cinnamic acid, and Trolox.

modifications. Briefly, ABTS•$^+$ was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate for 16 h in the dark at room temperature. The ABTS•$^+$ solution was diluted with MeOH to an absorbance of 0.70 ± 0.01 at 734 nm. In a glass 96-well reaction plate, samples along with positive (BHT, ascorbic acid, Trolox) and negative (cinnamic acid) controls (20 μL, 1 mg/mL, 0.02−1.0 mg/mL for Trolox) prepared in methanol were combined in triplicate with the ABTS•$^+$ solution (400 μL, absorbance 0.70 ± 0.01). After a brief incubation (6 min, 30 °C), the absorbance at 734 nm was read on a Molecular Devices Spectromax 384-Plus plate reader.

ORAC-Fluorescein Assay. Antioxidant capacity as assessed by the ORAC-fluorescein assay was accomplished following the methods of Prior et al. (*23*) and Davalos et al. (*24*) with some modifications. Trolox standards (1−10 μM), fluorescein (7.0 μM), and AAPH (63 mM) solutions were prepared prior to use in phosphate buffer (75 mM, pH 7.4). Limonin (1 mg/mL) and nomilin (1 mg/mL) were prepared in MeOH. Limonin glucoside (1 mg/mL), BHT (0.10 mg/mL), ascorbic acid (0.01 mg/mL), and cinnamic acid (1 mg/mL) were prepared in phosphate buffer. Blanks consisted of MeOH or phosphate. Sample (20 μL) and fluorescein (120 μL) solutions were placed in the well of a Costar 3631 assay plate (Corning Incorporated, Corning, NY). After incubation (37 °C, 15 min), AAPH (60 μL, 37 °C) was added rapidly using a multichannel pipet, and the microplate was immediately placed in a Molecular Devices Gemini-EM (Sunnyvale, CA) fluorescence plate reader. The plate was bottom-read (λ_{ex} = 485 nm and λ_{em} = 520 nm, 515-nm cutoff, 37 °C) at 1-min intervals for 80 min. Software supplied with the instrument was used to calculate the area under the fluorescence decay curve (AUC) and for subsequent data analysis. The net AUC corresponding to a sample was calculated by subtracting the AUC of the blank. A standard curve was generated from the net AUC of the Trolox standards and used to assign Trolox equivalence values to the samples.

β-Carotene−Linoleic Acid Antioxidant Activity Assay. On the basis of the methods described by Bouaziz et al. (*20*) and Montoro et al. (*25*), a microplate format assay was developed. β-Carotene (2 mg), linoleic acid (20 mg), and Tween 80 (200 mg) were combined in a round-bottom flask and suspended in $CHCl_3$ (5 mL). The chloroform was removed by rotary evaporation (40 °C), and an emulsion was formed by the addition of 50 mL (50 °C) of water. A control solution prepared in an identical manner, but lacking the β-carotene, was used to adjust the β-carotene-containing solution to an absorbance of 2.0−2.5 at 470 nm. Limonin (1 mg/mL), nomilin (1 mg/mL), limonin glucoside (1 mg/mL), BHT (1 mg/mL), ascorbic acid (10 mg/mL), and cinnamic acid (0.20 mg/mL) were prepared in MeOH and warmed to 50 °C prior to use. Blanks consisted of MeOH. Samples (325 μL) were combined with the β-carotene-containing emulsion (3.25 mL) and transferred rapidly (400 μL/per well) to a glass 96-well reaction plate using a multichannel pipet. The microplate was immediately placed in a Molecular Devices Spectromax 384-Plus (Sunnyvale, CA) plate reader maintained at 45°C, and the absorbance was read at 470 nm. The plate was read at 10-min intervals for the next 2 h. Between readings, the microplate was kept in a water bath at 50 °C. A control sample consisting of MeOH (325 μL) and the control solution (3.25 mL) was included on the microplate to account for the background absorbance. Analysis of the data was accomplished using the software provided with the instrument and Excel (Microsoft). Samples were corrected for background absorbance by subtracting the absorbance of the control sample and then normalized against the absorbance of the initial reading (time = 0 min) to reveal the decrease in absorbance over time.

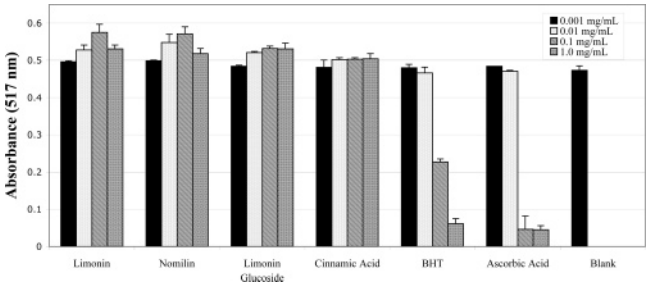

Figure 2. Antioxidant capacity of limonin, nomilin, limonin glucoside, cinnamic acid, BHT, and ascorbic acid as measured by the DPPH radical scavenging activity assay. Values are means ±SD, n = 3. Additional experimental details are described under Materials and Methods.

RESULTS

Four methods (ORAC, TEAC, β-carotene−linoleic acid bleaching, and DPPH radical scavenging assays) were used to evaluate the AOC of limonin, nomilin, and limonin glucoside. Both the TEAC and β-carotene−linoleic acid bleaching assays were adapted to microplate formats for convenience. Assays were conducted using pure limonoids and included cinnamic acid as the negative control and BHT and ascorbic acid as positive controls. For the DPPH radical scavenging assay the concentrations of the limonoids and controls were varied 0.001−1.0 mg/ mL. The limonoids and cinnamic acid were unable to quench the DPPH radical, whereas both ascorbic acid and BHT exhibited quenching activity at the 0.1 and 1.0 mg/mL concentrations (**Figure 2**). Results from DPPH radical scavenging assays are generally reported as IC_{50} values; however, since the limonoids were inactive this could not be done. Likewise when the same samples and controls were evaluated by the TEAC assay, only the positive controls exhibited a measurable AOC against the ABTS radical cation (**Figure 3**). Since the absorbance readings of the blank, cinnamic acid, and limonoids were not significantly different, it was not possible to calculate a Trolox equivalent for the cinnamic acid or limonoid samples. Trolox equivalents for limonin, nomilin, limonin glucoside, and cinnamic acid (**Table 1**) obtained from the ORAC-fluorescein

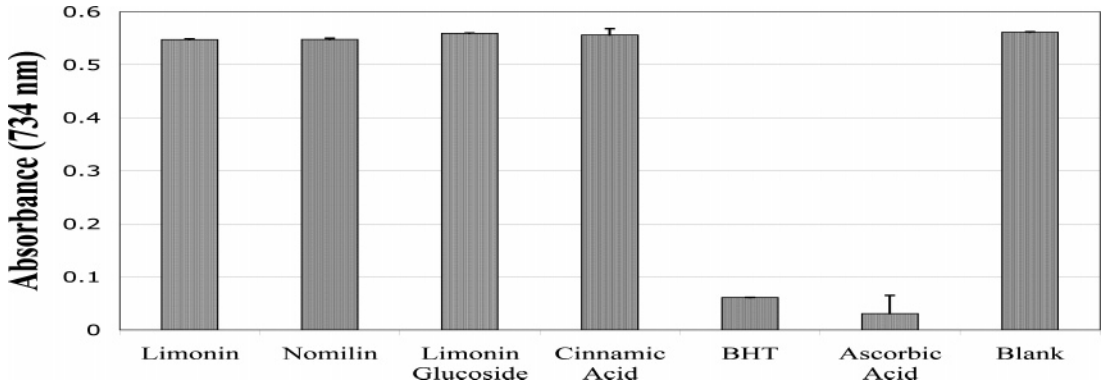

Figure 3. Antioxidant capacity of limonin, nomilin, limonin glucoside, cinnamic acid, BHT, and ascorbic acid as measured by the ABTS radical cation decolorization assay. Values are means ±SD, $n = 3$. Additional experimental details are described under Materials and Methods.

Table 1. Trolox Equivalents As Measured by the ORAC-Fluorescein Assay

sample	Trolox equiv[a,b]
limonin	0.034 ± 0.001
nomilin	0.030 ± 0.001
limonin glucoside	0.027 ± 0.001
cinnamic acid	0.027 ± 0.001
BHT	0.204 ± 0.002
ascorbic acid	1.528 ± 0.037

[a] Expressed as mmol Trolox equivalent/mmol pure compound. [b] Results presented as the mean ($n = 6$) ± SD.

assay were of the same order of magnitude and found to be approximately 7 times and 50 times less potent than BHT and ascorbic acid, respectively. **Figure 4** shows the results from the β-carotene−linoleic acid bleaching assay. The order of AOC obtained from this assay was BHT ≫ ascorbic acid ≫ limonin glucoside > limonin, nomilin, cinnamic acid, and blank. In initial experiments for this assay, we attempted to duplicate the ascorbic acid concentrations used by Sun et al. (*14*) but found a 10-fold increase in concentration was required to reach the level displayed in **Figure 4**. In addition, we found that cinnamic acid at concentrations above 0.2 mg/mL inhibited decolorization and at concentrations of 1.0 mg/mL was equivalent to BHT. This was the only assay in which any of the limonoids showed a capacity greater than that of the blank or the cinnamic acid negative control.

DISCUSSION

The growing awareness among consumers and the media that foods can provide more than nourishment has led to an explosion in the development of methods and reports of the analysis of foods and the constituents therein for evaluation of biological activities relevant to maintaining or improving human health and nutrition. Chief among these many assessments is the determination of antioxidant capacity (AOC) of abundant secondary metabolites in fruits and vegetables. However, the difficulties associated with comparing and reconciling numerous AOC reports indicated the lack of standardization of methods to address the nature of the samples and the sample matrixes. To address this issue the "First International Congress on Antioxidant Methods" was convened in 2004. The outcome of the meeting was a series of White Papers describing the various available AOC measuring methods and a set of guidelines for conducting AOC measurements (*15;16*). Recommendations included (1) that pure compounds be used so that any AOC or lack thereof could be attributed unambiguously to the compound under investigation and (2) that evaluation included both hydrogen atom transfer (HAT) and electron transfer (ET) mechanism based assays. Furthermore the oxygen radical absorbance capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) assays were recommended for adoption as the standard HAT and ET methods. Unstated in the White Papers but understood as the molecular basis of antioxidant activity among plant secondary metabolites are chemical structural

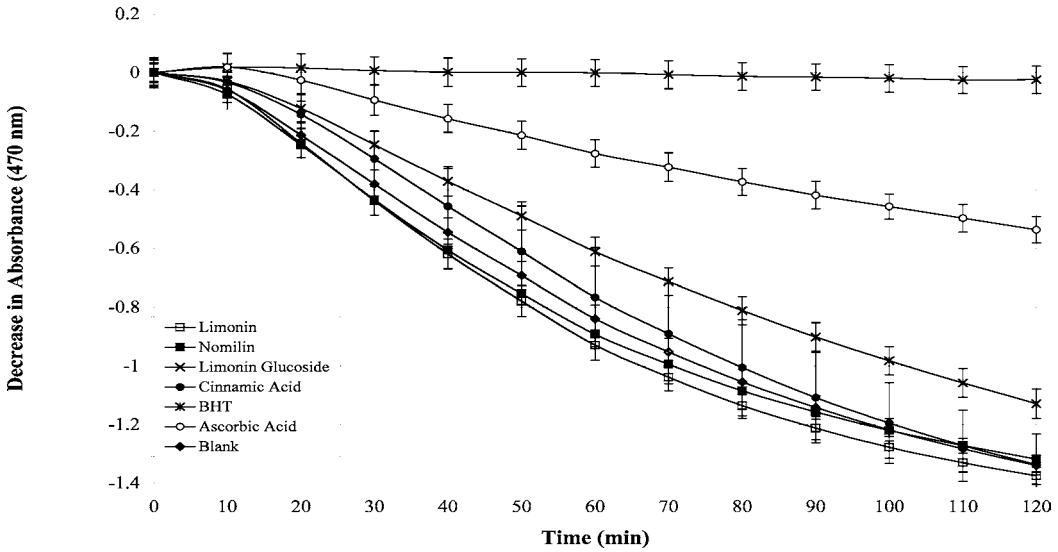

Figure 4. Antioxidant capacity of limonin, nomilin, limonin glucoside, cinnamic acid, BHT, and ascorbic acid as measured by the β-carotene–linoleic acid bleaching assay. Values are means ±SD, $n = 3$. Additional experimental details are described under Materials and Methods.

requirements including electron delocalization, redox, and free radical stabilization potential.

In this study, three representative limonoids (limonin, nomilin, and limonin glucoside) were chosen for evaluation on the basis of their structure, relative abundance, and the availability of pure samples. For most citrus species, limonin followed by nomilin are the most abundant aglycones, whereas the most abundant glucoside is limonin glucoside. Nomilin and limonin (**Figure 1**) are considered the initial and terminal points in the aglycone biosynthetic pathway. In the conversion of nomilin to limonin the B−C−D-ring structure remains intact while the biosynthetic effort focuses on the conversion of the A-ring of nomilin to the A−A′-ring structure of limonin (*26*). The majority of other aglycones isolated from citrus are intermediates possessing variations in the structure of the A-ring. Akin to the aglycones, the glucosides isolated thus far vary in the structure of their A-ring, while uniformly maintaining the same B−C-ring structure with the D-ring opened to a carboxylic acid and a single glucose moiety attached to the C-17 hydroxyl. Since the transition from nomilin to limonin does not result in the incorporation or the removal of a structural feature associated with antioxidant properties, nomilin and limonin, as the extremities of the biosynthetic pathway, were chosen to represent the limonoid aglycones and evaluate their AOC and the potential effect of the A-ring structure. Limonin glucoside was chosen as a representative to evaluate the effect of glycosylation on the AOC.

Following the recommendations of the White Papers, we have evaluated the AOC of pure limonin, nomilin, and limonin glucoside using the ORAC and TEAC assays. In addition, the limonoids were evaluated by the β-carotene−linoleic acid bleaching and DPPH radical scavenging assays. In all cases, limonin and nomilin gave results equivalent to those of cinnamic acid, indicating that they do not possess any inherent AOC and should not be considered antioxidants. Similar results were obtained for limonin glucoside, with the exception of the β-carotene−linoleic acid bleaching assay. The anomalous behavior of limonin glucoside in the β-carotene−linoleic acid bleaching assay is consistent with the suggestion by Prior et al. (*15*) that this method is of limited utility due to the lack of a standard format for obtaining and expressing results and that multiple pathways of β-carotene decolorization convolute the interpretation of results. Our observations support the statement of Prior et al., and in light of this we consider the limonin glucoside AOC results obtained by the β-carotene−linoleic acid bleaching assay to be inconclusive; we therefore conclude that limonin glucoside, like the other limonoids, should not be considered an antioxidant.

Prior to our study, AOC values reported for citrus limonoids and limonoid-containing extracts ranged from undetectable to greater than that for vitamin C (*11*−*14*). Among these reports, nomilinic acid glucoside (*12*) and limonin (*14*) were reported to have exhibited a significant AOC, the former approaching and the latter exceeding ascorbic acid. The limonoids used in both of these studies were impure. Poulose et al. (*12*) reported an estimated purity of 90% for their nomilinic acid glucoside sample, whereas the purity of the limonin obtained by Sun et al. (*14*) from a commercial source has a reported purity of 75% ± 5% according to the manufacturer's product description (Sigma Product No. 9647). The comparison of our AOC results utilizing pure limonoids with those of Poulose et al. and Sun et al. strongly suggests that impurities in the limonoid samples used in those studies may have been responsible for the AOC activity observed. The contrast of AOC evaluations on the limonoids serves to reinforce the relevance of purity in the determination of AOC (*15;16*).

Reports on the AOC of a number of natural products have suggested that a number of structural features are essential, including a phenol moiety or an extended conjugated system (*19;20;25;27*). Consideration of the structural character of citrus limonoids in light of the AOC chemical structure requirements reveals that this class of compounds lacks the molecular basis to possess AOC (**Figure 1**). Our experimental results confirm this premise.

ABBREVIATIONS USED

AOC, antioxidant capacity; ORAC, oxygen radical absorbance capacity; TEAC, Trolox equivalent antioxidant capacity; DPPH, 1,1-diphenyl-2-picryl hydrazyl; BHT, 2,6-di-*tert*-butyl-4-methylphenol; ABTS, 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; HAT, hydrogen atom transfer; ET, electron transfer.

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