Correlation between Flavonoid Content and the NO Production Inhibitory Activity of Peel Extracts from Various Citrus Fruits

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We investigated the correlation between the flavonoid content and NO production inhibitory activity of fruit peel extracts using 20 citrus plants. The contents of seven flavonoids (naringin, naringenin, hesperidin, hesperetin, rutin, nobiletin, and tangeretin) were determined by HPLC analysis. Each citrus peel extract varied in flavonoid content, but the contents of nobiletin and tangeretin, which were contained in all 20 fruit peels, showed a positive and significant correlation with each other (r=0.879, p<0.0005 for immature fruit peels; r=0.858, p<0.0005 for mature fruit peels). All citrus peel extracts dose-dependently inhibited LPS-induced NO production in RAW 264.7 cells. This inhibitory effect was significantly and positively correlated with the content of nobiletin and tangeretin. Nobiletin showed a more potent NO production inhibitory activity (IC50=26.5 μM) compared to tangeretin (IC50=136.6 μM). This result supports the premise that nobiletin-rich citrus may provide protection against disease resulting from excessive NO production.

Key words  citrus peel; flavonoid; nitric oxide (NO) production inhibitory activity; nobiletin

Plants of the genus Citrus are primarily valued for their edible fruit, but they also have traditional medicinal value. The peel of Citrus fruits has been used in traditional Asian medicine for centuries. Two traditional Chinese medicines, which are used to treat indigestion, zhi qiao and zhi shi, are obtained from the mature and immature fruit peels of Citrus aurantium L., respectively.1 In modern European herbal medicine, the fruit peel of C. aurantium is used to treat dyspepsia and related conditions.2,3 The dried peel of Citrus unshiu Marc. has been used as a traditional medicine in China and Japan to improve bronchial and asthmatic conditions or cardiac and blood circulation and is known as “Chinpi”.4

The peel of citrus fruits is a rich source of flavonones, as well as many polymethoxylated flavones, which are very rare in other plants.5 The most prevalent flavonones are hesperetin and naringenin, both of which are found in the fruit peel largely as their glycosides, hesperidin and naringin, respectively.5 Tangeretin and nobiletin are two polymethoxylated flavones that are commonly found in citrus fruit peels. These compounds not only play important physiological and ecological roles but are also of commercial interest because they have a multitude of applications in the food and pharmaceutical industries. For example, naringenin and hesperidin may act as antioxidants and anti-inflammatory agents.6—11 Polymethoxylated flavones are also of interest for their various pharmacological potentials, the most important of which are antitumor, anti-inflammatory, antimutagenic, and antiallergic properties.12—17 Rutin, a flavonol glycoside commonly found in citrus fruit peels, has been shown to have significant anti-inflammatory properties.18 In general, the contents and distributions of flavonoids in different Citrus species are highly variable and depend on genetic and environmental factors.1,19

The citrus flavonoids exhibit abilities to modulate the inflammatory response and carcinogenesis at a number of key regulatory points via several different mechanisms; thus, citrus fruits represent a potentially important source of anti-inflammatory flavonoids in the human diet.20 Nitric oxide (NO) has been implicated in a variety of pathophysiological conditions including inflammation, carcinogenesis, and atherosclerosis.21—28 The development of substances to prevent the overproduction of NO has become a new research target to treat chronic inflammatory diseases.24,29

Jeju Island in Korea is a unique place because citrus plants are cultivated on a large scale in the island’s subtropical climate. A better understanding of the relationship between the flavonoid content and NO production inhibitory effect may be useful in developing a program for the effective utilization of citrus fruit peels and flavonoids in human health. In this study, we investigated the correlation between the contents of flavonoids and the NO production inhibitory activities of fruit peels from 20 Citrus plants grown on Jeju Island.

MATERIALS AND METHODS

Materials  Nobiletin and tangeretin were obtained from Wako (Osaka, Japan). Other analytical-grade flavonoid standards and all other chemicals of analytical grade were purchased from Sigma (St. Louis, MO, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). LPS (Escherichia coli 026:B6), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and all other reagents, unless otherwise noted, were purchased from Sigma (St. Louis, MO, U.S.A.).

Plant Materials  Twenty species (including cultivars) of Citrus grown on Jeju Island, Korea were selected for fruit peel testing (Table 1). The tested citrus fruits were grown on Jeju Island in Korea is a unique place because citrus plants are cultivated on a large scale in the island’s subtropical climate. A better understanding of the relationship between the flavonoid content and NO production inhibitory effect may be useful in developing a program for the effective utilization of citrus fruit peels and flavonoids in human health. In this study, we investigated the correlation between the contents of flavonoids and the NO production inhibitory activities of fruit peels from 20 Citrus plants grown on Jeju Island.

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Table 1. *Citrus* Plants Investigated in This Study\(^\text{a}\)

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Korean name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. aurantium</em> L.</td>
<td>Jikak</td>
</tr>
<tr>
<td><em>C. benikoji</em> Hort. ex Tan.</td>
<td>Kamja</td>
</tr>
<tr>
<td><em>C. erythroa</em> Hort. ex Tan.</td>
<td>Dongjeongkooyol</td>
</tr>
<tr>
<td><em>C. grandis</em> (L.) Osb.</td>
<td>Dangyooya</td>
</tr>
<tr>
<td>*C. nippokorean Tan.</td>
<td>Cheongyoool</td>
</tr>
<tr>
<td><em>C. platymamma</em> Hort. ex Tan.</td>
<td>Byungkooyol</td>
</tr>
<tr>
<td><em>C. pseudogalga</em> Hort. ex Tan.</td>
<td>Sadookam</td>
</tr>
<tr>
<td><em>C. sunki</em> Hort. ex Tan.</td>
<td>Jinkyool</td>
</tr>
<tr>
<td><em>C. tachibana</em> (Marc.) Tan.</td>
<td>Hongkooyol</td>
</tr>
<tr>
<td><em>C. junos</em> Sieb. ex Tan.</td>
<td>Yooja</td>
</tr>
<tr>
<td><em>C. leioacarpa</em> Hort. ex Tan.</td>
<td>Binkyool</td>
</tr>
<tr>
<td><em>C. tangorina</em> Hort. ex Tan.</td>
<td>Pyunkyoool</td>
</tr>
<tr>
<td><em>C. natsudaidai</em> Hayata</td>
<td>Hakyoool</td>
</tr>
<tr>
<td><em>C. sulphata</em> Hort. ex Takahashi</td>
<td>Sambokam</td>
</tr>
<tr>
<td><em>C. depressa</em> Hayata</td>
<td>Shiikuwasha</td>
</tr>
<tr>
<td><em>C. iyo</em> Hort. ex Tan.</td>
<td>Iyekam</td>
</tr>
<tr>
<td><em>C. unshiu</em> Marc.</td>
<td>Josaeng</td>
</tr>
<tr>
<td><em>C. hassaku</em> Hort. ex Tan.</td>
<td>Palsak</td>
</tr>
<tr>
<td><em>C. unshiu</em> Marc. × <em>C. sinensis</em> (L.) Osb.</td>
<td>Cheungkooun</td>
</tr>
<tr>
<td><em>C. unshiu</em> × <em>C. sinensis</em> × <em>C. reticulata</em></td>
<td>Hallabong</td>
</tr>
</tbody>
</table>

\(^{a}\) The classification of *Citrus* plants are based on Tanaka’s system.

Fig. 1. Structures of the Flavonoids Investigated

through September 2005, and mature fruits were harvested from November 2005 through January 2006. The fruit peels were dissected, weighed, lyophilized, and ground with a mortar and pestle. The powdered peels were stored at \(-20\) °C prior to use.

**Sample Preparation** Portions (10 g) of the powdered peels were extracted for 3 d with 200 ml of ethanol–water (7: 3, v/v) at ambient temperature. The extract was decanted, and the remaining residue was extracted once more with 100 ml of the same solution. The combined extract was evaporated, lyophilized, and reconstituted with ethanol–water (1: 1, v/v) to a final concentration of 100 mg/ml.

**Flavonoid Analysis** The structures of seven flavonoids analyzed in this study are shown in Fig. 1. Reconstituted samples were filtered through a membrane filter (0.45 μm). A 10 μl aliquot of filtered sample was injected into a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, U.S.A.) equipped with a pump, UV–vis detector, column oven, and injector. A C\(_18\) RP column (Atlantis dc-18, 150×3.9 mm-i.d.; Waters) with a cartridge guard column was used for the HPLC system. The mobile phase for the HPLC system was acetonitrile (A) and water (B) with a flow rate of 1 ml/min. The mobile phase program consisted of four periods: 0–10 min, 20% A; 10–16 min, 45% A; 16–20 min, 75% A; and 20–22 min, 20% A. The column was operated at 40 °C, and the eluent was monitored with a single-channel UV detector at a wavelength of 280 nm. The flavonoids were identified by comparing their retention times and UV spectra with those of authentic standards stored in a data processor. The content of each flavonoid was calculated from the integrated peak area of the sample and the corresponding standard.

**Cell Culture** The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in DMEM medium with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were supplemented with 10% FBS in a humidified atmosphere with 5% CO\(_2\) at 37 °C.

**Nitrite Assay** To evaluate the inhibitory activity of the peel extracts on LPS-induced NO production, cells were plated in 96-well plates (5×10\(^4\) cells/well) and incubated for 24 h. Cells were then treated with LPS (100 ng/ml) in the presence or absence of test materials. After an additional 24 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction.\(^30\) The percent (%) inhibition was expressed as (1–(NO level of test samples/NO level of vehicle-treated control))×100. The IC\(_{50}\) value, equivalent to the sample concentration that inhibits NO production by 50%, was determined using nonlinear regression analysis (% inhibition versus concentration).

**MTT Assay** Cell viability was determined by the MTT assay.\(^31\) After the cells were cultured as described above, the MTT solution (final 0.5 mg/ml) was added to each well and further incubated for 1 h at 37 °C. Media were discarded, and dimethyl sulfoxide (DMSO) was added to each well to dissolve the generated formazan. The absorbance was measured at 570 nm, and the percentage survival was determined by comparison with the control group.

**Statistical Analysis** Data were presented as the mean± (relative) standard deviation, and all experiments were conducted in triplicate. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, U.S.A.). Correlations between variables were analyzed using Spearman’s correlation of rank coefficient. Wilcoxon’s signed-ranks test or paired t-test was used to determine the statistical significance. \(p<0.05\) was considered significant.

**RESULTS**

**Distributions of the Flavonoids among Various Citrus Fruit Peels** The flavonoid contents of the immature and mature fruit peel extracts derived from the 20 citrus species are presented in Tables 2 and 3, respectively. Hesperidin was widely distributed in large amounts in the peels of both immature and mature citrus fruit, whereas naringenin was present at high levels in only specific citrus fruits, such as *C. aurantium*, which also contained rutin at the highest amount among the 20 citrus species. Naringenin and hesperetin, the
aglycones of naringin and hesperidin, were distributed in lower concentrations in the citrus fruit peels. Nobiletin and tangeretin occurred ubiquitously in most citrus species with similar distribution patterns. The correlation analysis between flavonoid contents revealed that the nobiletin content was positively and significantly correlated with the tangeretin content in peel extracts of both the immature ($r=0.879$, $p<0.0005$) and mature ($r=0.858$, $p<0.0005$) citrus fruits (Table 4). However, no significant correlation was detected among the contents of the other flavonoids (Table 4). The contents of naringin, hesperidin, nobiletin, and tangeretin in the peels of mature citrus fruits were significantly less than those of immature citrus fruits (Fig. 2).

### NO Production Inhibitory Effects of the Fruit Peel Ex-
were considered to be the most potent NO production inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5). A marked difference was observed in inhibitory activity on LPS-induced NO production in RAW 264.7 cells (Table 5).

**Table 4. Correlation between Flavonoid Content in the Peel Extracts of Citrus Fruits**

<table>
<thead>
<tr>
<th>Citrus fruit peel</th>
<th>Naringin</th>
<th>Hesperidin</th>
<th>Naringenin</th>
<th>Hesperetin</th>
<th>Rutin</th>
<th>Nobiletin</th>
<th>Tangeretin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naringin</td>
<td>-0.259</td>
<td>1.000</td>
<td>0.119</td>
<td>0.378</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hesperidin</td>
<td>0.285</td>
<td>0.257</td>
<td>0.103</td>
<td>0.400</td>
<td>0.173</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.376</td>
<td>0.257</td>
<td>0.103</td>
<td>0.400</td>
<td>0.173</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Hesperetin</td>
<td>-0.070</td>
<td>0.103</td>
<td>0.400</td>
<td>0.173</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rutin</td>
<td>-0.193</td>
<td>0.060</td>
<td>0.225</td>
<td>0.019</td>
<td>-0.147</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Nobiletin</td>
<td>-0.138</td>
<td>0.139</td>
<td>0.213</td>
<td>0.125</td>
<td>-0.135</td>
<td>0.879***</td>
<td>1.000</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>-0.044</td>
<td>n.c.</td>
<td>0.197</td>
<td>-0.139</td>
<td>-0.015</td>
<td>0.858***</td>
<td>1.000</td>
</tr>
</tbody>
</table>

***p<0.0005, n.c.; not calculated.

**Fig. 2.** Box Plots of the Naringin, Hesperidin, Nobiletin, and Tangeretin Contents in the Peel Extracts of Immature and Mature Citrus Fruits

The flavonoid content was calculated from the integrated peak area of the sample and the corresponding standard. The box represents the interquartile range, the line within the box designates the median, and the dotted line denotes the arithmetic mean. The ends of the ‘whiskers’ show the maximum and minimum values. *p<0.05, **p<0.005 by Wilcoxon’s signed-ranks test.

**Table 5. Correlation between Flavonoid Content and NO Production Inhibitory Activity**

<table>
<thead>
<tr>
<th>Citrus fruit peel</th>
<th>Naringin</th>
<th>Hesperidin</th>
<th>Naringenin</th>
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<th>Rutin</th>
<th>Nobiletin</th>
<th>Tangeretin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
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<td></td>
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<td></td>
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<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Nobiletin</td>
<td>-0.138</td>
<td>0.139</td>
<td>0.213</td>
<td>0.125</td>
<td>-0.135</td>
<td>0.879***</td>
<td>1.000</td>
</tr>
<tr>
<td>Tangeretin</td>
<td>-0.044</td>
<td>n.c.</td>
<td>0.197</td>
<td>-0.139</td>
<td>-0.015</td>
<td>0.858***</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Correlation between Flavonoid Content and NO Production Inhibitory Activity**

The NO production inhibitory activity of citrus fruit peels was significantly and positively correlated with both nobiletin and tangeretin contents at all tested concentrations (Table 6). In accordance with these results, the IC$_{50}$ values were always negatively correlated with both nobiletin and tangeretin contents (Table 6). However, no significant correlation was found among the other flavonoid contents and respective NO-production inhibitory activities.

To define the extent of each flavonoid’s contribution to the potential anti-inflammatory activity, the NO production inhibitory efficacy of each flavonoid (naringin, hesperidin, naringenin, hesperetin, rutin, nobiletin, and tangeretin) was compared to each other in LPS-activated RAW 264.7 cells (Fig. 4). Among the tested flavonoids, nobiletin was found to be the most potent NO production inhibitor (IC$_{50}=26.5$ μM). In comparison, the IC$_{50}$ value of tangeretin was found to be 136.6 μM. This result indicates that the correlation between the tangeretin content and NO production inhibitory activity shown in Table 6 could have been caused by the highly positive correlation between the nobiletin and tangeretin contents of the citrus fruit peels.

**Correlation between Flavonoid Content and NO Production Inhibitory Activity**

Citrus fruit-derived flavonoids and their metabolites have been shown to have significant biological activities, including anti-inflammatory properties. A large body of evidence implicates flavonoids extracted from citrus fruit peels as being the most bioactive as supported by the high biological activities of peel components compared to fruit juice sac components.

Among the seven flavonoids analyzed in this study, hesperidin was widely distributed with a varying amount in the majority of the tested citrus fruit peels, whereas naringin was present at high levels in only certain citrus fruits such as *C. aurantium*. Both immature and mature fruit peels of *C. aurantium* contained the highest amounts of naringin and rutin. Similar results was documented by Nogata et al., they have analyzed 17 flavonoids from 46 citrus samples. It is known that the immature and mature fruit peels of *C. aurantium* are used to treat indigestion in traditional Chinese medicine and modern European herbal medicine.

Naringenin, a metabo-
lite of naringin, has been shown in many studies to prevent gastric mucosal ulceration in several animal models, including restraint stress, pyloric occlusion, and ethanol-induced chronic ulceration.\(^{35-38}\) It has been reported that naringin also possesses antioxidant and superoxide anion scavenger properties that could contribute to its gastro-protective effect.\(^{39,40}\) We suggest that the fruit peel of \(C. \textit{aurantium}\) is an excellent source for the isolation of naringin to fulfill potential industrial and pharmacological applications. Hesperidin was ubiquitously contained to varying degrees in the peels of both immature and mature fruits from all of the citrus varieties tested except mature \(C. \textit{natsudaidai}\) \textit{Hayata}.

![Table 5. Inhibitory Activity of Citrus Fruit Peel Extracts on NO Production in LPS-Activated RAW 264.7 Cells](image)

<table>
<thead>
<tr>
<th>Citrus</th>
<th>Peel of immature citrus fruits</th>
<th>Peel of mature citrus fruits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu g/ml): 250 500 1000 IC(_{50})</td>
<td>(\mu g/ml): 250 500 1000 IC(_{50})</td>
</tr>
<tr>
<td>(C. \textit{aurantium}) L.</td>
<td>—</td>
<td>13.2 33.7 (a) 6.3 14.5 13.7 (a)</td>
</tr>
<tr>
<td>(C. \textit{benthoji}) \textit{Hort. ex Tan.}</td>
<td>25.2 54.8 92.6 443.3</td>
<td>14.7 24.4 41.8 (a)</td>
</tr>
<tr>
<td>(C. \textit{erythrosum}) \textit{Hort. ex Tan.}</td>
<td>15.7 34.1 66.1 716.6</td>
<td>9.6 22.0 36.6 (a)</td>
</tr>
<tr>
<td>(C. \textit{grandis}) (L.) \textit{Osib.}</td>
<td>6.3 21.6 50.6 996.5</td>
<td>7.6 18.3 32.4 (a)</td>
</tr>
<tr>
<td>(C. \textit{nippokoreana}) \textit{Tan.}</td>
<td>—</td>
<td>27.4 59.9 745.6 22.2 23.0 53.9 930.7</td>
</tr>
<tr>
<td>(C. \textit{platymamma}) \textit{Hort. ex Tan.}</td>
<td>41.6 71.8 92.8 303.0</td>
<td>23.4 37.6 72.1 631.9</td>
</tr>
<tr>
<td>(C. \textit{pseudogalgal}) \textit{Hort. ex Tan.}</td>
<td>—</td>
<td>— 19.6 (a)</td>
</tr>
<tr>
<td>(C. \textit{suki}) \textit{Hort. ex Tan.}</td>
<td>64.8 88.9 96.8 188.4</td>
<td>15.4 42.3 71.1 580.9</td>
</tr>
<tr>
<td>(C. \textit{tachibana}) (\textit{Marck.}) \textit{Tan.}</td>
<td>51.7 79.8 93.9 239.9</td>
<td>11.1 22.0 52.2 945.9</td>
</tr>
<tr>
<td>(C. \textit{juvens}) \textit{Siv. ex Tan.}</td>
<td>—</td>
<td>18.4 34.1 (a) 11.6 21.6 33.0 (a)</td>
</tr>
<tr>
<td>(C. \textit{leioecarpus}) \textit{Hort. ex Tan.}</td>
<td>22.5 52.6 81.5 477.3</td>
<td>19.6 33.9 56.7 768.3</td>
</tr>
<tr>
<td>(C. \textit{tangerina}) \textit{Hort. ex Tan.}</td>
<td>24.9 51.2 93.4 476.5</td>
<td>12.3 17.4 37.3 (a)</td>
</tr>
<tr>
<td>(C. \textit{natsudaiai}) \textit{Hayata}</td>
<td>—</td>
<td>— 7.2 29.1 (a)</td>
</tr>
<tr>
<td>(C. \textit{scleata}) \textit{Hort. ex Takahashi}</td>
<td>45.9 85.6 95.4 259.8</td>
<td>15.2 48.6 70.2 521.6</td>
</tr>
<tr>
<td>(C. \textit{depressa}) \textit{Hayata}</td>
<td>6.0 24.1 45.5 (a)</td>
<td>13.4 26.2 37.9 (a)</td>
</tr>
<tr>
<td>(C. \textit{iyo}) \textit{Hort. ex Tan.}</td>
<td>—</td>
<td>— 9.0 27.9 (a)</td>
</tr>
<tr>
<td>(C. \textit{unshiu}) \textit{MARC.}</td>
<td>45.9 85.6 95.4 259.8</td>
<td>15.2 48.6 70.2 521.6</td>
</tr>
<tr>
<td>(C. \textit{psuedogalgal}) \textit{Hort. ex Tan.}</td>
<td>6.0 24.1 45.5 (a)</td>
<td>13.4 26.2 37.9 (a)</td>
</tr>
<tr>
<td>(C. \textit{natsudaiai}) \textit{Hayata}</td>
<td>—</td>
<td>— 9.0 27.9 (a)</td>
</tr>
<tr>
<td>(C. \textit{tangerina}) \textit{Hort. ex Tan.}</td>
<td>24.9 51.2 93.4 476.5</td>
<td>12.3 17.4 37.3 (a)</td>
</tr>
<tr>
<td>(C. \textit{natsudaiai}) \textit{Hayata}</td>
<td>—</td>
<td>— 7.2 29.1 (a)</td>
</tr>
</tbody>
</table>
| Data are presented as average of inhibitory activity (%) (\(n=3\)); In all cases the standard deviation is less than 10% of the average. —; inhibitory activity \(<5\%\), \(a\) \(IC_{50}>1000\mu g/ml\).

Fig. 3. Box plots of the Inhibitory Activity of LPS-Induced NO Production by the Peel Extracts of Immature and Mature Citrus Fruits

Cells were treated with LPS (100 ng/ml) in the presence or absence of citrus fruit peel extracts at the indicated concentrations for 24h. The amount of nitrite released in the culture medium was determined by the Griess reagent. The box represents the interquartile range, the line within the box designates the median, and the dotted line indicates the arithmetic mean. The ends of the ‘whiskers’ show the maximum and minimum values. \(*p<0.05\), \(*\*p<0.005\) by Wilcoxon's signed-ranks test.

Table 6. Correlation between Flavonoid Content and Inhibitory Activity on LPS-Induced NO Production in Citrus Fruit Peel Extracts

<table>
<thead>
<tr>
<th>Citrus fruit peel</th>
<th>Concentration ((\mu g/ml))</th>
<th>Naringin</th>
<th>Naringenin</th>
<th>Hesperidin</th>
<th>Hesperetin</th>
<th>Rutin</th>
<th>Nobiletin</th>
<th>Tangeretin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Immature})</td>
<td>250</td>
<td>—0.135</td>
<td>0.181</td>
<td>0.070</td>
<td>0.067</td>
<td>—0.436</td>
<td>0.869****</td>
<td>0.811***</td>
</tr>
<tr>
<td>500</td>
<td>—0.232</td>
<td>0.179</td>
<td>0.228</td>
<td>0.074</td>
<td>—0.245</td>
<td>0.942****</td>
<td>0.870***</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>—0.170</td>
<td>0.179</td>
<td>0.244</td>
<td>0.180</td>
<td>—0.251</td>
<td>0.923****</td>
<td>0.881***</td>
<td></td>
</tr>
<tr>
<td>(IC_{50})</td>
<td>0.195</td>
<td>—0.077</td>
<td>0.236</td>
<td>0.041</td>
<td>0.614</td>
<td>—0.907****</td>
<td>—0.787**</td>
<td></td>
</tr>
<tr>
<td>(\text{Mature})</td>
<td>250</td>
<td>—0.331</td>
<td>n.c.</td>
<td>0.168</td>
<td>—0.219</td>
<td>—0.226</td>
<td>0.671**</td>
<td>0.576*</td>
</tr>
<tr>
<td>500</td>
<td>—0.282</td>
<td>n.c.</td>
<td>0.135</td>
<td>—0.259</td>
<td>—0.267</td>
<td>0.762****</td>
<td>0.605**</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>—0.361</td>
<td>n.c.</td>
<td>—0.002</td>
<td>—0.378</td>
<td>—0.237</td>
<td>0.901****</td>
<td>0.763***</td>
<td></td>
</tr>
<tr>
<td>(IC_{50})</td>
<td>0.158</td>
<td>n.c.</td>
<td>—0.054</td>
<td>n.c.</td>
<td>0.223</td>
<td>—0.500</td>
<td>—0.429</td>
<td></td>
</tr>
</tbody>
</table>

\(*p<0.05\), \(*\*p<0.005\), \(*\*\*p<0.0005\), n.c.: not calculated.
Both nobiletin and tangeretin also showed ubiquitous distributions among the peels of both immature and mature fruits from all the citrus varieties except Citrus junos Sieb. ex Tan. We found that the nobiletin content was correlated significantly and positively with the tangeretin content. In general, the total amount of flavonoids within the mature citrus fruit peels was significantly lower than those of the immature fruit peels, confirming that the flavonoid contents in citrus fruit peels change dramatically during maturation.

The majority of the citrus fruit peels suppressed LPS-induced NO production in RAW 264.7 cells, although the inhibitory activities varied. The NO-production inhibitory activities of the mature fruit peels were significantly lower than those of the immature fruit peels, suggesting that the NO production inhibitory activity of a citrus fruit peel can be determined by the flavonoid composition, which was unique to each citrus plant. Notably, the NO production inhibitory activities of the citrus fruit peels were correlated with both the nobiletin and tangeretin contents.

By comparing the NO production inhibitory activities of the flavonoids, we found that nobiletin was the most potent among the tested flavonoids (Fig. 4). Unexpectedly, however, the NO production inhibitory activity of tangeretin was about five times lower than that of nobiletin, suggesting that the positive correlation observed between tangeretin content and NO production inhibitory activity may have been merely due to the high positive correlation between the nobiletin and tangeretin contents in citrus fruit peels. Nobiletin is a polymethoxylated flavone occurring exclusively in Citrus plants that has been reported to be a promising anti-inflammatory and antitumor agent. Nobiletin has been found to exhibit anti-inflammatory activity on phorbol ester-induced skin inflammation in mice and to produce a more potent protective activity than indomethacin in a TPA-induced, edema formation test in mouse ears.12,13 Recently, nobiletin was shown to inhibit eosinophilic airway inflammation in asthmatic rats.14 Citrus nobiletin also inhibits azoxymethane-induced large bowel carcinogenesis in rats and down-regulates matrix metalloproteinase-7 expression in HT-29 human colorectal cancer cells.15,16

In conclusion, the flavonoid contents and NO production inhibitory effects of the peel extracts investigated in this study were unique to each citrus plant. Among the seven flavonoids analyzed, the nobiletin content and NO production inhibitory effect of each citrus fruit peel were highly correlated. This finding suggests various beneficial features of citrus fruit peels such as the prevention of diseases that involve excessive NO release.

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