Marker Constituents of the Natural Antioxidant Eucalyptus Leaf Extract for the Evaluation of Food Additives

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Received November 26, 2008; Accepted February 9, 2009; Online Publication, May 7, 2009
[doi:10.1271/bbb.80832]

In order to establish the marker constituents of the natural antioxidant food-additive Eucalyptus leaf extract, the UV-absorbing constituents of two eucalyptus leaf extracts registered as food additives (eucalyptus A and B) were investigated. Several major peaks on the reversed-phase HPLC chromatogram of eucalyptus A were characterized as gallic acid, ellagic acid, 3-O-β-D-glucuronolactone of quercetin and kaempferol, and a hudrolyzable tannin dimer, oenothein B, by direct comparison with authentic specimens isolated from Eucalyptus globulus leaves. A new gallotannin was found in the leaf extract, including the antioxidative activity by radical scavenging ability, a standardization of degradation products from hydrolyzable tannins in the leaves. Considering the evaluation of antioxidant activity by radical scavenging ability, a standardization of eucalyptus leaf extract, including the antioxidative polyphenol, oenothein B, is proposed.

Key words: natural antioxidant; food additive; eucalyptus; Eucalyptus globulus; polyphenol

Natural antioxidant food additives, most of which are extracts including numerous ingredients, are widely used in food processing to prevent deterioration in the quality of foods by oxidative damage. Among a variety of such natural additives registered in the List of Existing Food Additives in Japan,¹ eucalyptus leaf extract is defined as an ethanol extract or steam distillate from the leaves of Eucalyptus globulus Labill (Myrtaceae), and this additive is characterized as containing β-diketones as the main constituents.

E. globulus has traditionally been used as a medicinal plant for the treatment of enterocolitis, arthralgia, and burns in China.² Eucalyptus oils prepared by steam distillation are also used as flavoring and fragrance.³ β-Diketones, such as 16-hydroxy-18-tritriacantone and 4-hydroxy-tritriacantane-16,18-dione (wax constituents), as well as ellagic acid and its related compounds, were reported as antioxidants of the leaves of E. globulus by Osawa et al. in the 1980s.⁴-⁶ In addition to the antioxidants, the chemical constituents of the leaves have been investigated extensively to reveal the presence of a wide array of compounds, including monoterpenes, such as 1,8-cineole, sesquiterpenephloroglucinol derivatives, flavonoids, tannins, and related polyphenols.³⁷-¹³

As part of studies evaluating official food additives conducted by research groups of the Ministry of Health, Labour and Welfare of Japan since 2003, eucalyptus leaf extract was found to include gallic acid, quercetin 3-O-β-D-glucuronide, kaempferol 3-O-β-D-glucuronide, globulinside, cryptmeridiol, 4-epi-cryptmeridiol, 3β,13β-dihydroxyurs-11-en-28-oic acid, β-ecdysone, and macarocalp I, of which the first three were the main constituents.¹⁴ However, β-diketones, defined in the official list as the main constituents, were not detected. Thus the standardized markers in eucalyptus leaf extract are currently undefined.

This paper describes a re-examination of the constituents of the commercial food additive eucalyptus leaf extract based on a detailed characterization of the antioxidant polyphenols of the raw material (E. globulus leaves). It also proposes a new standard, including a characteristic marker ingredient, for the quality of this antioxidant food additive.

Materials and Methods

General. UV spectra were recorded on a Shimadzu UVmini-1240 (Shimadzu, Kyoto, Japan). Electrospray ionization (ESI)-MS and high-resolution (HR) ESI-MS spectra were obtained using a microTOF-Q (Bruker Daltonics, Billerica, MA) mass spectrometer using acetone/tetrahydrofuran/tetramethylsilane as the solvent. H- and C-NMR spectra were recorded on a Bruker AVANCE500 instrument (Bruker BioSpin, Billerica, MA) (500 MHz for H and 126 MHz for C), and chemical shifts are given in ppm values relative to those of the solvents (methanol-d₄ (δH 3.30; δC 49.0) and acetone-d₆ (δH 2.04; δC 49.0)) on a tetramethylsilane scale. The standard pulse sequences programmed for the instrument (Avance 500) were used for the 2D measurements (COSY, HSQC, and HMBC). JCH was set at 8 or 10 Hz in HMBC.

Reversed-phase (RP) HPLC conditions were as follows: column, Cosmosil packed column SCL8-PAQ (5 μm, 150 × 4.6 mm i.d.) (Nacalai Tesque, Kyoto, Japan); mobile phase, solvent A was 5% acetic acid and solvent B was methanol (MeOH) (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85% B in A; 40–50 min, 95–90%; 50–55 min, 90–100% B in A; 55–60 min, 100% B); column temperature, 40 °C; flow rate, 1.0 ml/min; detection, 200–400 nm. Normal-phase HPLC conditions were as follows: column, YMC-Pack SIL A-002 (5 μm, 150 × 4.6 mm i.d.); mobile phase, n-hexane-MeOH-tetrahydrofuran-formic acid (55:33:11:1) and...
oxalic acid 450 mg/l; column temperature, room temperature; flow rate, 1.5 ml/min; detection, 280 nm.

Column chromatography was conducted using MCI Gel CHP-20P (75–150 μm) (Mitsubishi Chemical, Tokyo), Toyopearl HW-40 (fine grade) (Tosoh, Tokyo), and YMC GEL ODS-AQ with aqueous MeOH to give (7 g), which showed many peaks on HPLC similar to those of the food.

**Samples and reagents.** Two commercial eucalyptus leaf extract products (eucalyptus A: brown powder, eucalyptus B: brown liquid) were obtained through the Japan Food Additives Association (JFA) (Tokyo). Three commercial eucalyptus oils were used as products derived from *E. globulus*. The samples were dissolved in MeOH or aqueous MeOH before HPLC and TLC. Fresh leaves of *E. globulus* were collected from the medicinal plant garden of Matsuyama University in April 2007. Dried leaves were kindly donated by Nagaoka Perfumery Co., Ltd. (Osaka, Japan).

**Extraction and isolation.** Dried leaves of *E. globulus* (400 g) were homogenized in 70% MeOH (MeOH·H₂O 7:3) (10 liters), and a concentrated solution (about 1 liter) was extracted successively with n-hexane (5 liters), ethyl acetate (5 liters), and water-saturated n-butanol (5 liters) to give the respective n-hexane (4.2 g), ethyl acetate (7.0 g), and water (3.2 g) extracts. The ethyl acetate extract (7 g) showed many peaks on HPLC similar to those of the food additive products, was chromatographed over Toyopearl HW-40, MCI-GEL CHP-20P, and YMC GEL ODS-AQ with aqueous MeOH to give a new compound, 1,2,3,6-tetra-O-galloyl-β-D-galactose (14) (2 mg), together with gallic acid (1) (15 mg), oenothein B (2) (34 mg), ellagic acid (3) (20 mg), quercetin 3-O-β-D-glucuronide (4) (86 mg), kaempferol 3-O-β-D-glucoside (5) (53 mg), chlorogenic acid (6) (38 mg), 5-O-p-coumaroylquinic acid (7) (8 mg), chlorogenic acid (8) (26 mg), quercetin 4′-O-β-D-glucoside (9) (13 mg), trimagallin (10) (5 mg), trimagallin II (11) (18 mg), 1,2,3-tri-O-galloyl-β-D-glucose (12) (3 mg), 1,2,3,4,6-penta-O-galloyl-β-D-glucose (13) (2 mg), 8-demethylsideroxyl (16) (2 mg), sideroxyl (17) (2 mg), and 3′-O-methyl ellagitannin 4-O-β-D-glucose (18) (2 mg). These compounds were identified by direct comparison with authentic specimens or by comparison of their spectral data with those reported in the literature.

**Results**

**HPLC analysis of eucalyptus leaf extract**

Two commercial eucalyptus leaf extract products (eucalyptus A and B) used as antioxidant food additives were analyzed. Figure 1 shows their HPLC chromatograms at 270 nm. Eucalyptus A showed a HPLC profile
(Fig. 1a) similar to that (Fig. 1c) of an aqueous MeOH extract prepared from the dried leaves of *E. globulus*, the raw material of eucalyptus A and B, while eucalyptus B gave a much simpler chromatogram showing only two main peaks (Fig. 1b). On the other hand, eucalyptus oil did not show the same peaks (Fig. 1d). In order to examine standard samples for the characterization of each HPLC peak in Fig. 1, an aqueous MeOH extract was separated by column chromatography, and 18 compounds (1–18) were characterized, as shown in Fig. 2. Among them, compound 14 was a new compound and its structure was elucidated as follows: The molecular formula of 14, C_{34}H_{28}O_{22}, was determined by HR-ESI-MS in negative mode (m/z 787.0974 [M – H]−, calcd. 787.1000). The 1H-NMR spectrum showed signals attributable to four galloyl groups (δ 7.07, 7.06, 7.05, and 6.95, each 2H-singlet), and aliphatic proton signals characteristic of a galactopyranose residue of C1 conformation. The constituent units of 14 were determined by acid hydrolysis, yielding galactose and gallic acid, the former of which was proven to be a D-series by HPLC analysis of the derivative of the sugar by the method reported by Tanaka et al.29) The positions of the galloyl groups on the galactose residue were assigned as O-1, O-2, O-3, and O-6 based on the appearance of the H-1–H-3 and H-6 signals in the lower field (δ 6.08–4.43) than the H-4 signal (δ 4.36). The β-orientation of the O-1 galloyl group was indicated by a large coupling constant (d, J = 8.5 Hz) of the anomeric proton signal. Based on these spectroscopic data together with 13C-NMR and 2D NMR (HSQC and HMBC) spectra, 14 was characterized.
as 1,2,3,6-tetra-O-galloyl-β-D-galactose. Since most gallotannins characterized to date have a D-glucose core, the presence of a galactose core in 14 is a rare example in nature.

Based on a comparison with the HPLC data of the above isolates from *E. globulus*, the main HPLC peaks of eucalyptus A were characterized as gallic acid (1), oenothein B (2), ellagic acid (3), quercetin 3-O-β-D-glucuronide (4), kaempferol 3-O-β-D-glucuronide (5), and chlorogenic acid (6). On the other hand, the two main peaks in eucalyptus B were identified as 1 and 3.

Although 1 and 3 have been reported frequently as natural products, they are considered to be mostly artifacts produced from hydrolyzable tannins during the drying and/or isolation procedures. In fact, upon treatment of eucalyptus A with hot water monitoring by HPLC, 1 and 3 increased time-dependently (Fig. 3), and the HPLC after 8 h was similar to that of eucalyptus B. Eucalyptus B was thus considered to be a product prepared by heat extraction of the leaves.

**TLC analysis of eucalyptus leaf extracts**

β-Diketones in *E. globulus* have been reported to give spots at RF 0.7–0.8 on silica gel TLC developed with *n*-hexane-ethyl acetate (3:1) under UV irradiation. In accordance with the previous finding, neither eucalyptus A nor B showed such spots on TLC. Extracts of *E. globulus* leaves were prepared with CHCl₃, EtOH, and 50% aqueous EtOH. β-Diketones were not detected by TLC with the 50% EtOH extract prepared from fresh or dried leaves of *E. globulus* (Fig. 4 (1)), while the CHCl₃ and EtOH extracts of the dried leaves exhibited spots at about RF about 0.8 under UV light and after spraying with dilute sulfuric acid followed by heating the plate (Fig. 4 (2)).

**Antioxidant properties of eucalyptus leaf extract**

The antioxidant effects of the food additives (eucalyptus A and B) in comparison with those of the CHCl₃, EtOH, and 50% EtOH extracts from fresh and dried leaves of *E. globulus* were assessed by DPPH radical scavenging assay and the ORAC method. As shown in Table 1, eucalyptus A showed the most potent antioxidant activity in DPPH radical scavenging assay, comparable to that of the 50% EtOH extract. Conversely, eucalyptus B exhibited extremely weak activity, similar to that of the CHCl₃ extract of the dried leaves, although these effects might have been underestimated because they are oily products. The ORAC values showed similar results (Fig. 5).

Because the eucalyptus leaf extract registered as a food additive in the “List of Existing Food Additives in...
Japan’ ‘ includes a product by steam distillation, three commercially available eucalyptus oils (A–C) were also examined for antioxidant properties by DPPH radical scavenging assay. As expected, these oils showed no antioxidant effect (EC50 > 100 mg/ml, Table 1).

Discussion

Besides the previously reported major constituents (1, 4, and 5) of the food additive eucalyptus leaf extract, compounds 2 and 3 were newly detected as other major components of eucalyptus A, which showed a potent antioxidative effect in this study. Oenothein B (2) is a unique hydrolyzable tannin dimer with a macrocyclic structure that is known to exhibit diverse biological activities, such as anti-tumor, antiviral, and a restoration effect of antibiotics toward methicillin-resistant Staphylococcus aureus (MRSA) as well as antioxidant effects.30) Although 2 has been found in some species of Eucalyptus, Eugenia, and Melaleuca genera of Myrtaceae,31–33) this study is the first report of its isolation from E. globulus.

The antioxidant potencies of eucalyptus A and B as well as the other extracts with various solvents were clearly explained by their total polyphenol contents, as seen in Table 1. The HPLC and TLC chromatograms of eucalyptus A together with the relation between antioxidant effect and total polyphenols were similar to those of the aqueous alcohol extract of the dried leaves of E. globules (Figs. 1 and 4), implying that eucalyptus A is a product produced by aqueous EtOH extraction at ambient temperature. On the other hand, the similarity of the HPLC profiles between eucalyptus B and the partial hydrolyzate from the 50% EtOH extract suggests that eucalyptus B was produced by heat extraction (Figs. 1b and 3).

The present study reconfirms that β-diketones were not detected in the eucalyptus leaf extracts, in agreement with a previous paper.14) Although CHCl3 extracts of the dried leaves indicated distinct spots (Rf about 0.8) assignable to β-diketones or related non-polar substances on TLC (Fig. 4 (2)), these substances might not contribute to the antioxidative effect, because the CHCl3 extract had extremely weak antioxidant properties on both radical scavenging and ORAC assay. Hence, it is suggested that polyphenols should be defined as the major components of the antioxidant food additive eucalyptus leaf extract in the “List of Existing Food Additives in Japan” rather than β-diketones.

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Based on the results of the present study, eucalyptus leaf extract, as prepared by extraction with 50% EtOH but not by steam distillation, can be characterized as a polyphenol-rich antioxidant additive with potency stronger than that of ascorbic acid (ORAC, 500–1,000 μmol TE/g).34,35) Among polyphenols, including flavonol glycosides and hydrolyzable tannins characterized in the extract, bioactive oenothein B (2) might be a significant marker characteristic of the E. globulus leaf, because it is a rare natural product with a unique

<p>| Table 1. DPPH Radical Scavenging Activity of Eucalyptus Leaf Extracts and Eucalyptus Oils |
|----------------------------------|-------------------|------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>EC50a (μg/ml)</th>
<th>Total polyphenolsb (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucalyptus A</td>
<td>19.0</td>
<td>360</td>
</tr>
<tr>
<td>Eucalyptus B</td>
<td>390.0</td>
<td>29</td>
</tr>
<tr>
<td>E. globulus (fresh leaves)</td>
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<td></td>
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<tr>
<td>Chloroform extract</td>
<td>162.7</td>
<td>34</td>
</tr>
<tr>
<td>EtOH extract</td>
<td>23.5</td>
<td>237</td>
</tr>
<tr>
<td>50%aq. EtOH extract</td>
<td>19.1</td>
<td>548</td>
</tr>
<tr>
<td>E. globulus (dried leaves)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform extract</td>
<td>477.0</td>
<td>30</td>
</tr>
<tr>
<td>EtOH extract</td>
<td>44.1</td>
<td>357</td>
</tr>
<tr>
<td>50%aq. EtOH extract</td>
<td>24.7</td>
<td>408</td>
</tr>
<tr>
<td>Eucalyptus Oil A</td>
<td>&gt; 100 mg/ml</td>
<td>29</td>
</tr>
<tr>
<td>Eucalyptus Oil B</td>
<td>&gt; 100 mg/ml</td>
<td>—</td>
</tr>
<tr>
<td>Eucalyptus Oil C</td>
<td>&gt; 100 mg/ml</td>
<td>—</td>
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</tbody>
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*aEC50, 50% effective concentration
bThese values are expressed as mg of gallic acid equivalent per g of samples.

Fig. 5. ORAC Values of Eucalyptus Leaf Extracts. TE, Trolox equivalent
structure and can be detected easily without interference from other peaks on a reversed-phase HPLC chromatogram in a gradient mode with 5% AcOH and MeOH (Fig. 1). In addition, 2 is fairly stable, as was observed upon heating eucalyptus A (Fig. 3). Hence, compound 2 might be a good standard constituent for evaluating eucalyptus leaf extract. On the ORAC assay, which is widely used in the USA to evaluate the antioxidant effects of foods and related natural products, eucalyptus leaf extract was found to be a long-lasting antioxidant with a potency of about 4,000 μmol TE/g (Fig. 5), which confirms its usefulness as a natural antioxidant food additive.

Acknowledgments

The authors thank Ms. Mie Tokuhara of Matsuyama University, for technical assistance. Thanks are also extended to the Japan Food Additives Association for providing eucalyptus leaf extracts, and to Nagaoka Perfumery Co., Ltd., for donating dried leaves of *E. globulus*. This work was supported by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan.

References