Note

Hydrolyzable Tannins as Antioxidants in the Leaf Extract of *Eucalyptus globulus* Possessing Tyrosinase and Hyaluronidase Inhibitory Activities

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Received October 20, 2008; Accepted January 28, 2009

The aqueous ethanolic extract of *Eucalyptus globulus* leaves has been used as an antioxidant in food additives. From the extract, five ellagitannins and four gallotannins were isolated as the major antioxidative components. Heterophyllin A, 1,3-di-*O*-galloyl-4,6-hexahydroxydiphenoyl- β -D-glucose, 1,2,4-tri-*O*-galloyl- β -D-glucose, 1,2,3,6-tetra-*O*-galloyl- β -D-glucose and 1,2,4,6-tetra-*O*-galloyl- β -D-glucose are the first to be isolated from this plant. These hydrolyzable tannins exhibited more potent antioxidant activity (superoxide dismutase-like activity) than ascorbic acid, as well as inhibitory activities against tyrosinase and hyaluronidase. Thus, the eucalyptus leaf extract may be useful in food and cosmetics for not only antioxidation, but also anti-melanogenesis, anti-allergy and anti-inflammation.

Keywords: *Eucalyptus globulus*; hydrolyzable tannin; superoxide anion radical scavenging activity; tyrosinase inhibitory activity; hyaluronidase inhibitory activity.

Introduction

The evergreen tree *Eucalyptus globulus* Labill (Myrtaceae) native to Australia is widely distributed around the world. The leaves, bark and fruit of this plant have been externally used as traditional remedies for inflammation and incision in China (Okuda, 1986), while its leaves, from which tea is made, have been used as a traditional medicine for diabetes in South America and Africa (Gray and Flatt, 1998).

The essential oil distilled from the eucalyptus leaves, containing the major component 1,8-cineol, is industrially produced and consumed in great quantities as in throat drops, flavor, fragrances, and aromatherapy. The aqueous ethanolic extract of the leaves (eucalyptus leaf extract: ELE) is used as one of the natural food additives for antioxidation in Japan (The List of Existing Food Additives; Japanese Ministry of Health and Welfare, 1999). Some potent antioxidative com-

pounds, such as β -diketone (16-hydroxy-18-tritriacontanone and 4-hydroxy-tritriacontane-16,18-dione) and ellagic acid, have also been found in this plant (Osawa and Namiki, 1985; Amakura *et al.*, 2002); however, details about the composition profile of the antioxidants involved in the ELE used as a food additive have not yet been well elucidated.

In the present study, we isolated five ellagitannins and four gallotannins, along with their aglycones, as the major antioxidative components from the ELE through fractionations guided by the superoxide anion radical scavenging activity (superoxide dismutase (SOD)-like activity). In addition, we investigated their inhibitory effects on tyrosinase (EC 1.14.18.1) and hyaluronidase (EC 3.2.1.35).

Materials and Methods

Material, Extraction and Purification The dried leaves of *E. globulus* (500 g), which were harvested in Spain and purchased from K. Kobayashi & Co., Ltd. (Kobe, Japan), were extracted with 40% EtOH (3 L) under reflux for 2 h.

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After cooling and filtration, the filtrate was concentrated *in vacuo* and lyophilized to give an extract (ELE). The ELE solubilized in water (0.3 L) was then successively extracted with Et₂O, EtOAc and *n*-BuOH (each 0.9 L), and each organic layer was evaporated to dryness *in vacuo* to give the respective extracts and the remaining water fraction which was freeze-dried. To isolate the antioxidative components, the *n*-BuOH (24.4 g) and EtOAc (8.7 g) extracts were further fractionated by column chromatography over Diaion HP-20 (Mitsubishi Chemical Industry, Tokyo, Japan), TSK-gel HW-40 (fine grade; Tosoh, Tokyo, Japan) and MCI-GEL CHP-20P (37–75 μ m; Mitsubishi Chemical Industry) with a stepwise increase in the EtOH concentration in water. The components of each fraction were monitored by absorbance at 280 nm using normal-phase HPLC on a Inertsil SIL column (250 mm $\times$ 4.6 mm i.d.; GL Sciences, Inc., Tokyo, Japan) and reversed-phase HPLC on a Inertsil ODS column (150 mm $\times$ 4.6 mm i.d.; GL Sciences) with a UV detector as previously described (Okuda *et al.* 1989). A portion (18 g) of the *n*-BuOH extract was applied to a Diaion HP-20 column (3.3 cm i.d. $\times$ 30 cm) and eluted with an increasing concentration gradient of aqueous EtOH (0% $\rightarrow$ 20% $\rightarrow$ 40% $\rightarrow$ 60% $\rightarrow$ 100% EtOH). Only the 20% EtOH eluate (5.9 g, EC₅₀: 0.73 μ g/mL) showed the SOD-like activity stronger than the crude extract, and was further separated by column chromatography over Toyopearl HW-40 with EtOH-H₂O (5:5 $\rightarrow$ 6:4 $\rightarrow$ 10:0) $\rightarrow$ 70% acetone. The fractions showing similar HPLC patterns were combined. The most active fractions (EC₅₀: 0.29 μ g/mL) from the 20% EtOH eluate were combined and purified by repeated column chromatography over MCI-gel CHP-20P with aqueous EtOH (0% $\rightarrow$ 10% $\rightarrow$ 20% $\rightarrow$ 30% EtOH) to give compounds **1** (30 mg) and **2** (10 mg). The EtOAc extract (5 g) was subjected to column chromatography over Toyopearl HW-40 (2.2 cm i.d. $\times$ 30 cm) to yield compound **10** as the precipitate (86 mg). Among the eluate of EtOH-H₂O (5:5), fractions show-

ing low EC₅₀ (0.50–1.14 μ g/mL) were combined and further chromatographed over Toyopearl HW-40 and MCI-gel CHP-20P to give compounds **11** (90 mg), **6** (3 mg) and **7** (2 mg). The eluates of EtOH-H₂O (6:4 and 7:3, EC₅₀: 1.09–1.21 μ g/mL, respectively) were similarly fractionated by repeated column chromatography over MCI-gel CHP-20P to afford compounds **2** (251 mg), **4** (3 mg), **8** (4 mg), **9** (20 mg), **3** (5 mg) and **5** (3 mg). ¹H- and ¹³C-NMR spectra were recorded on a Jeol JNM a-500 NMR (¹H, 500 MHz; ¹³C, 126 MHz) or Varian VXR-600 spectrometer (¹H, 600 MHz) in acetone-*d*₆ containing D₂O. Chemical shifts were given in δ ppm with tetramethylsilane (TMS) as an internal standard.

Determination of SOD-like activity The test samples were dissolved in 10% dimethylsulfoxide (DMSO) for evaluation of the SOD-like activity according to the NBT method (Amakura *et al.*, 2002) using xanthine oxidase (from bovine milk; Sigma) to generate the superoxide anion radical.

Determination of tyrosinase inhibition test The inhibitory activity for tyrosinase (from mushroom; Sigma) was determined by the dopachrome method (Matsuda *et al.*, 1994). Briefly, the sample solution prepared in 10% DMSO was added to 1/15 M phosphate buffer (pH 6.8) containing 50 μ g/mL 3-(3,4-dihydroxyphenyl)-L-alanine. The reaction mixture was preincubated for 10 min at 25°C. The change in absorbance at 475 nm was followed for 5 min after adding 300 units/mL mushroom tyrosinase.

Determination of hyaluronidase inhibition test The test samples were dissolved in 10% DMSO for determination of the hyaluronidase activity by the modified Morgan-Elson method. The sample solution was mixed with hyaluronidase (400 units/mL; from bovine testis Type IV-S; Sigma) and compound 48/80 (0.1 mg/mL; Sigma) containing hyaluronic acid potassium salt (0.4 mg/mL; from rooster comb; Sigma) in 0.1 M acetate buffer (pH 4.0) at 37°C for 40 min to prepare the reaction mixture (Ono *et al.*, 2001).

Table 1. Superoxide anion radical scavenging activity (SOD-like activity) and tyrosinase and hyaluronidase inhibitory effects on the ELE.

Sample	SOD-like activity (EC ₅₀ : μ g/ml)	Tyrosinase inhibition (IC ₅₀ : mg/ml)	Hyaluronidase inhibition (IC ₅₀ : mg/ml)
ELE	1.02	0.39	1.05
Et ₂ O extract	4.90	>1.00	>2.00
EtOAc extract	0.96	0.61	0.14
<i>n</i> -BuOH extract	0.77	0.32	0.69
H ₂ O residue	1.38	0.83	1.63

Results and Discussion

The ELE showed a strong SOD-like activity with an EC_{50} value of 1.02 $\mu\text{g/mL}$ (Table 1). After separation using organic solvents, the *n*-BuOH and EtOAc extracts were further fractionated to isolate eleven compounds in the ELE with antioxidant activity (Fig. 1). The *n*-BuOH extract provided two hydrolyzable tannins, which were identified as pedunculagin (1) (Yoshida *et al.*, 1984) and tellimagrandin I (2) (Yoshida *et al.*, 1984). The EtOAc extract yielded tellimagrandin I (2), II (3) (Wilkins and Bohm, 1976), het-

erophyllin A (4) (Yoshida *et al.*, 1991), 1,3-di-*O*-galloyl-4,6-hexahydroxydiphenoyl (HHDP) - β -D-glucose (5) (Yoshida *et al.*, 1992), 1,2,4-tri-*O*-galloyl- β -D-glucose (6) (Hussein *et al.*, 1997), 1,2,3,6-tetra-*O*-galloyl- β -D-glucose (7) (Haddock *et al.*, 1982), 1,2,4,6-tetra-*O*-galloyl- β -D-glucose (8) (Haddock *et al.*, 1982), 1,2,3,4,6-penta-*O*-galloyl- β -D-glucose (9) (Haddock *et al.*, 1982), ellagic acid (10) and gallic acid (11). Compound 6 was identified by its corresponding published spectroscopic data. Other compounds were identified by direct comparison of their UV, $^1\text{H-NMR}$ and/or $^{13}\text{C-NMR}$

(1)

(2): $R^1 = R^2 = \text{H}$, $R^3 = \text{G}$

(3): $R^1 = \text{OG}$, $R^2 = \text{H}$, $R^3 = \text{G}$

(4): $R^1 = R^3 = \text{H}$, $R^2 = \text{OG}$

(5): $R^1 = \text{OG}$, $R^2 = R^3 = \text{H}$

(6): $R^1 = R^3 = \text{G}$, $R^2 = R^4 = \text{H}$

(7): $R^1 = R^2 = R^4 = \text{G}$, $R^3 = \text{H}$

(8): $R^1 = R^3 = R^4 = \text{G}$, $R^2 = \text{H}$

(9): $R^1 = R^2 = R^3 = R^4 = \text{G}$

(10)

(11)

Fig. 1. Chemical structures of the antioxidative components isolated from the ELE.

spectral data with those from actual specimens (Okuda *et al.*, 1995).

This is the first report on the isolation of **4–8** from *E. globulus*. Their $^1\text{H-NMR}$ spectral data are as follows.

Heterophylliin A (**4**), $^1\text{H-NMR}$ (500 MHz, acetone- d_6 containing D_2O) δ ppm: 7.24, 7.04 (each 2H, s, galloyl-H), 6.62, 6.48 (each 1H, s, HHDP-H), 6.41 (1H, d, $J=3.5$ Hz, glucose H-1), 5.65 (1H, t, $J=10$ Hz, H-3), 5.25 (1H, dd, $J=6.5$, 13.5 Hz, H-6), 5.06 (1H, t, $J=10$ Hz, H-4), 4.52 (1H, dd-like, $J=6.5$, 10 Hz, H-5), 4.21 (1H, dd, $J=3.5$, 10 Hz, H-2), 3.76 (1H, d, $J=13.5$ Hz, H-6).

1,3-Di-*O*-galloyl-4,6-HHDP- β -D-glucose (**5**), $^1\text{H-NMR}$ (500 MHz, acetone- d_6 containing D_2O) δ ppm: 7.19, 7.03 (each 2H, s, galloyl-H), 6.61, 6.47 (each 1H, s, HHDP-H), 5.87 (1H, d, $J=8.5$ Hz, glucose H-1), 5.46 (1H, dd, $J=9.5$, 10.5 Hz, H-3), 5.28 (1H, dd, $J=6.5$, 13 Hz, H-6), 5.04 (1H, t, $J=10.5$ Hz, H-4), 4.32 (1H, dd-like, $J=6.5$, 10 Hz, H-5), 3.98 (1H, dd, $J=8.5$, 9.5 Hz, H-2), 3.81 (1H, d-like, $J=13$ Hz, H-6).

1,2,4-Tri-*O*-galloyl- β -D-glucose (**6**), $^1\text{H-NMR}$ (500 MHz, acetone- d_6 containing D_2O) δ ppm: 7.19, 7.03 (each 2H, s, galloyl-H), 6.07 (1H, d, $J=8.5$ Hz, glucose H-1), 5.59 (1H, t, $J=9.5$ Hz, H-4), 5.38 (1H, dd, $J=8.5$, 9.5 Hz, H-2), 3.96 (1H,

t, $J=9.5$ Hz, H-3), 3.91 (1H, dd, $J=2$, 12 Hz, H-6), 3.80 (1H, dd, $J=5$, 12 Hz, H-6), 3.78 (1H, ddd, $J=2$, 5, 9.5 Hz, H-5).

1,2,3,6-Tetra-*O*-galloyl- β -D-glucose (**7**), $^1\text{H-NMR}$ (500 MHz, acetone- d_6 containing D_2O) δ ppm: 7.14, 7.07, 7.06, 6.99 (each 2H, s, galloyl-H), 6.11 (1H, d, $J=8$ Hz, glucose H-1), 5.66 (1H, t, $J=9.5$ Hz, H-3), 5.46 (1H, dd, $J=8$, 10 Hz, H-2), 4.64 (1H, dd, $J=2$, 12 Hz, H-6), 4.47 (1H, dd, $J=5$, 12 Hz, H-6), 4.14 (1H, ddd, $J=2$, 5.5, 10 Hz, H-5), 4.07 (1H, t, $J=9.5$ Hz, H-4).

1,2,4,6-Tetra-*O*-galloyl- β -D-glucose (**8**), $^1\text{H-NMR}$ (500 MHz, acetone- d_6 containing D_2O) δ ppm: 7.15, 7.14, 7.10, 7.07 (each 2H, s, galloyl-H), 6.05 (1H, d, $J=8.5$ Hz, glucose H-1), 5.41 (1H, t, $J=9.5$ Hz, H-3), 5.39 (1H, dd, $J=8.5$, 10 Hz, H-2), 4.54 (1H, dd, $J=2$, 13 Hz, H-6), 4.37 (1H, t, $J=9.5$ Hz, H-4), 4.30 (1H, ddd, $J=2.5$, 5, 10 Hz, H-5), 4.21 (1H, dd, $J=5$, 13 Hz, H-6).

Among these compounds, **1–5** are classified as ellagitannins and **6–9** as gallotannins. Compounds **10** and **11** are aglycones of these hydrolyzable tannins, with a significant part of **10** and **11** being artificially produced during the course of extraction under reflux and concentration of the extract *in vacuo*; that is, the HHDP and galloyl groups in the hydrolyzable tannins are readily liberated by physical and

Table 2. Superoxide anion radical scavenging activity (SOD-like activity) and tyrosinase and hyaluronidase inhibitory effects of the compounds isolated from the ELE.

Compounds	Molecular	SOD-like activity (EC_{50} : μM)	Tyrosinase inhibition (IC_{50} : mM)	Hyaluronidase inhibition (IC_{50} : mM)
1	784.6	0.18	0.48	1.51
2	786.6	0.34	0.11	0.90
3	938.7	0.74	0.35	0.58
4	786.6	0.35	0.30	0.89
5	788.6	0.22	0.35	0.74
6	636.5	0.25	0.95	1.57
7	788.6	0.18	0.32	0.35
8	786.6	1.55	0.59	0.68
9	940.7	0.98	0.24	0.55
10	302.2	2.71	>1.00	4.66
11	170.1	1.21	>1.00	5.00
Ascorbic acid	176.1	2.61	0.31	N.D.
DSCG	512.3	N.D.	N.D.	0.45

N.D.: not determined.

Each value is the mean of three experiments.

chemical actions (Amakura *et al.*, 2000).

These isolated compounds were examined for their SOD-like activity, and their EC₅₀ values were compared to that of ascorbic acid. As shown in Table 2, all of the hydrolyzable tannins isolated from the ELE indicated more potent SOD-like activity (EC₅₀, 0.18–1.55 µM) greater than that of ascorbic acid (EC₅₀, 2.61 µM).

The polyphenol oxidase tyrosinase causes enzymatic browning of foods such as fruits, leading to food deterioration (Friedman, 1996). It is also a key enzyme in melanogenesis of skin (Hearing and Jimenez, 1989). The superoxide anion radical facilitates tyrosinase activity (Valverde *et al.*, 1996), while certain antioxidants including ascorbic acid inhibit it (Briganti *et al.*, 2003). As the inhibitory effects of hydrolyzable tannins on tyrosinase activity have not been clarified, we examined the hydrolyzable tannins isolated from the ELE for their tyrosinase inhibitory activity. All of the isolated compounds acted as a tyrosinase inhibitor; in particular, the IC₅₀ of **2**, the most abundant antioxidative component isolated from the ELE, was substantially lower than that of ascorbic acid (Table 1). In contrast, their aglycones (**10** and **11**) showed no inhibition on tyrosinase, despite their EC₅₀ values indicating comparable or lower SOD-like activity than that of ascorbic acid.

Hyaluronidase, which decomposes hyaluronan, is implicated in allergies and inflammation, and some inhibitors of hyaluronidase have been useful for the suppression of these symptoms (Kakegawa *et al.*, 1985a). As tannins also have hyaluronidase inhibitory effects (Kakegawa *et al.*, 1985b; Lee *et al.*, 1993), the isolated compounds in this study were assayed for their antihyaluronidase activities. Among the tested compounds, **3**, **7** and **9** indicated effective IC₅₀ values comparable to that of disodium cromoglycate (DSCG) (Kobayashi *et al.*, 2004) as the control.

In summary, this study reports 11 hydrolyzable tannins as the major antioxidative components in ELE. These hydrolyzable tannins act as tyrosinase and hyaluronidase inhibitors, suggesting that the ELE is a useful additive not only for antioxidation in food, but also for anti-melanogenesis, anti-allergy and anti-inflammation in food and cosmetics.

Acknowledgement We are grateful to Prof. A. Masuyama (Faculty of Engineering, Osaka Institute of Technology, Osaka, Japan) for the NMR analysis.

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