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Constituents and their antioxidative effects in eucalyptus leaf extract used as a natural food additive

Yoshiaki Amakura[a,*], Yukiko Umino[a], Sumiko Tsuji[a], Hideyuki Ito[b], Tsutomu Hatano[b], Takashi Yoshida[b], Yasuhide Tonogai[a]

[a]*National Institute of Health Sciences, Osaka Branch, 1-1-43, Hoenzaka, Chuo-ku, Osaka 540-0006, Japan*
[b]*Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1, Tsushima, Okayama 700-8530, Japan*

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Abstract

The components of a natural food additive, "eucalyptus leaf extract", were isolated and identified in order to determine their structures and contents. The structures of eight major compounds, namely gallic and ellagic acids, eucalyptone and macrocarpals A–E, isolated from them were elucidated by spectroscopic methods. The antioxidative activities of these isolated compounds were estimated by several assays, and it appears that the antioxidant activity is mostly due to the gallic and ellagic acids. On the other hand, in the determined eucalyptus product, the content of 1,8-cineole, a major component of the eucalyptus oil, was lower than the isolated compounds, and its activity as an antioxidant was negligible.

Keywords: Eucalyptus leaf extract; Natural antioxidant; Food additive; NMR; HPLC

1. Introduction

In Japan, natural food additives have recently been included within the range of the designated system. These are analogous to synthetic chemical food additives, though thus far, only synthetics have been specified [Notification No. 101 (24 May 1995), Ministry of Health and Welfare, Japan; Yoshihira, 1996]. Therefore, an execution of the appropriate standardization for natural food additives is rapidly required, being important for health and food hygiene. Additionally, in view of international coordination, it is also deemed important. Based on this background, although many studies in this area are in progress (Goda, 1994; Yoshihira, 1996), most have still not yielded results and they are difficult because many natural food additives are found in plant materials as their complexes. Therefore, to begin with the included ingredients, particularly with major compounds and activities, one must clarify standards for the natural food additives.

The natural food additive, "eucalyptus leaf extract", is included as one of the antioxidants in the List of Existing Food Additives [Notification No. 120 (16 April 1996), Ministry of Health and Welfare, Japan]. In the list, its description as a food additive is merely "polyprenol, cineole, etc.", and the composition during its manufacture has not been investigated in detail.

This paper deals with the elucidation of the included constituents isolated from this food additive and their antioxidative activities based on several antioxidative assays.

2. Materials and methods

2.1. Samples and reagents

The commercial eucalyptus leaf extract (referred to as eucalyptus product) was obtained as a sample through the Japan Food Additive Association. This product was a brown liquid with the slightly characteristic smell and taste of eucalyptus. MCI-gel CHP-20P (37–75 μm) was purchased from the Mitsubishi Chemical Corporation (Tokyo, Japan). Gallic acid monohydrate, ellagic acid dihydrate, and 1,8-cineole were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Eucalyptone and

* Corresponding author at present address: National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Fax: +81-3-3707-6950.
E-mail address: amakura@nihs.go.jp (Y. Amakura).

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macrocarpals A–E, which were isolated in this experiment and then identified by comparison of the spectral data with those in the literature, were used as standards. Other chemicals were of HPLC or reagent grade. The standards were dissolved in methanol or ethanol.

2.2. NMR and MS analyses

The ^{1}H and ^{13}C NMR spectra were measured on Varian VXR-500 or 200 instruments (500 MHz for ^{1}H and 126 or 50 MHz for ^{13}C) in acetone-d_6 containing D_2O (ca. 3%) or pyridine-d_5. Chemical shifts are given in δ values (ppm) based on the chemical shifts of the solvent signals on a tetramethylsilane (TMS) scale in acetone-d_6 (δ_H 2.04, δ_C 29.8) or downfield from TMS in pyridine-d_5 as internal reference. The electrospray ionization (ESI)-MS results were recorded using a Shimadzu QP-8000 mass spectrometer with 50% aqueous acetonitrile as a solvent, and full scan spectra from m/z 100 to 1500 u in the negative mode were obtained.

2.3. HPLC conditions

The HPLC analysis were carried out using a Shimadzu class LC-VP HPLC system with class LC-VP software, a pump (LC-10Advp), an autosampler (SIL-10AD), and a diode-array detection (SPD-M10Avp) (Shimadzu, Kyoto, Japan). The UV spectrum was recorded between 200 and 400 nm.

The analytical conditions were as follows: column, LiChrosphere 100 RP-18 (5 μm, 4.6 mm i.d.×250 mm) (Merck, Darmstadt, Germany); mobile phase, solvent A was 5% acetic acid and B was methanol (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85–85% B in A; 40–50 min, 85–90% B in A; 50–55 min, 90–100% B in A; 55–70 min, 100% B); column temperature, 40 °C; flow-rate, 1.0 ml/min; detection, 270 nm; injection volume, 10 μl. The quantitative determination was carried out using linear calibration graphs obtained from each standard solution in the concentration range of 0.1–100 μg/ml.

Preparative conditions included the following. Method 1 (P1): column, YMC-Pack ODS-AQ (5 μm, 20 mm i.d.×250 mm) (YMC Co., Ltd., Kyoto, Japan); mobile phase, solvent A was 5% acetic acid and B was methanol (0–40 min, 30–80% B in A; 40–45 min, 80% B); column temperature, 40 °C; flow-rate, 4.0 ml/min; detection, 270 nm; injection volume, 150 μl. Method 2 (P2): mobile phase, methanol–acetic acid–water (120:2:13); flow-rate, 5.0 ml/min. Others were the same as those of method 1.

2.4. GC/MS conditions

The GC/MS analysis was carried out using a Shimadzu QP-5050 (Shimadzu, Kyoto, Japan). GC conditions were: column, DB-5 column (0.25 mm i.d.×30 m, 0.25 μm film thickness) (J&W Scientific, Rancho Cordova, CA); carrier gas, helium (1.7 ml/min); temperatures: detector, 260 °C; injector, 260 °C; oven programmed from an initial temperature, 50 °C (1 min hold) to 260 °C at 5 °C/min (5 min hold). MS conditions were: interface temperature, 260 °C; detector voltage, 1.0 kV; injection volume, 2 μl. Quantitative determination, monitored at m/z 154 by selected-ion monitoring (SIM), was carried out using linear calibration graphs obtained from a standard solution of 1,8-cineole diluted with *n*-hexane in the concentration range of 0.1–100 μg/ml.

2.5. Sample preparation for quantitative analysis

The eucalyptus product was dissolved in methanol at the concentration of 20 mg/ml. A 10-μl aliquot was subjected to analytical HPLC. On the other hand, the eucalyptus product (10 g) was extracted with *n*-hexane (10 ml×4), and then the extract was evaporated and the residue was adjusted to 10 ml with *n*-hexane. A 2-μl aliquot of the prepared solution was subjected to GC/MS.

2.6. Determination of total polyphenols

The total phenolic content was determined with Folin–Ciocalteu reagent according to the method of Julkunen-Tiitto (1985), using gallic acid as a standard. The result was expressed as milligrammes of gallic acid equivalent per gramme of the eucalyptus product.

2.7. Isolation of compounds

The eucalyptus product (50 g) was successively extracted with *n*-hexane (50 ml×4), ethyl acetate (50 ml×4) and *n*-butanol (50 ml×4). The *n*-hexane extract (0.6 g) was submitted to column chromatography over MCI-gel CHP-20P (11 mm i.d.×350 mm) with water containing increasing amounts of methanol in a stepwise gradient mode (70–90% methanol) to give eucalyptone (**3**) (2.5 mg), macrocarpals A (**4**) (5.0 mg) and C (**8**) (16.0 mg). The ethyl acetate extract (1.0 g) was subjected to MCI-gel CHP-20P (11 mm i.d.×350 mm) with aqueous methanol (30–90%), then purified by preparative HPLC (P1 and 2) to give gallic acid (**1**) (2.0 mg), ellagic acid (**2**) (6.5 mg), eucalyptone (**3**) (10.4 mg), macrocarpals A (**4**) (10.1 mg), B (**5**) (5.7 mg), D (**6**) (3.2 mg) and E (**7**) (3.5 mg). The *n*-butanol extract (5.0 g) was chromatographed on MCI-gel CHP-20P (11 mm i.d.×350 mm) with aqueous methanol (10–50%), and further purified by preparative HPLC (P1) to give gallic acid (**1**) (14.2 mg) and ellagic acid (**2**) (12.6 mg). The compounds were identified by direct comparison with authentic specimens or by comparisons of their physicochemical data with those reported in the literature. Among them, the ^{1}H NMR and MS spectral data were as follows.

- *Gallic acid (**1**)*: Off-white needles, ESI-MS *m/z*: 169 $[M-H]^-$, 339 $[2M-H]^-$. ^1H NMR (200 MHz, acetone-d_6 + D_2O) δ: 7.10 (2H, s).
- *Ellagic acid (**2**)*: Dark yellow amorphous powder, ESI-MS *m/z*: 301 $[M-H]^-$. ^1H NMR (500 MHz, acetone-d_6 + D_2O) δ: 7.58 (2H, s).
- *Eucalyptone (**3**)*: Pale yellow powder, ESI-MS *m/z*: 485 $[M-H]^-$. ^1H NMR (500 MHz, pyridine-d_5) δ: 0.59 (1H, br d, J = 7 Hz, H-6), 0.63 (1H, d, J = 7 Hz, H-7), 0.95 (6H, d, J = 6.6, H-12′, 13′), 1.10 (3H, s, H-13), 1.15 (3H, s, H-12), 1.41 (1H, dd, J = 13, 4 Hz, H-10′), 1.44 (3H, s, H-15), 1.48 (1H, m, H-11′), 1.75 (1H, dd, J = 10, 4 Hz, H-3α), 1.78 (1H, dd, J = 9.5, 6.5 Hz, H-8), 2.07 (3H, s, H-14), 2.14 (1H, ddd, J = 10, 10, 2 Hz, H-3β), 2.36 (1H, m, H-8), 2.37 (1H, ddd, J = 10, 10, 4 Hz, H-2α), 2.50 (1H, d, J = 7 Hz, H-5), 2.54 (1H, dd, J = 9.5, 6.5 Hz, H-9), 2.68 (1H, m, H-2β), 2.71 (1H, m, H-9), 2.72 (1H, dd, J = 13, 4 Hz, H-10′), 3.51 (1H, m, H-9′), 10.49 (1H, s, H-7′), 10.51 (1H, s, H-8′).
- *Macrocarpal A (**4**)*: Pale yellow powder, ESI-MS *m/z*: 471 $[M-H]^-$. ^1H NMR (500 MHz, pyridine-d_5) δ: 0.67 (3H, ddd, J = 9.5, 9.5, 6.5 Hz, H-7), 0.73 (3H, dd, J = 9.5, 9.5 Hz, H-6), 0.98 (3H, dd, J = 7 Hz, H-13′), 1.02 (3H, s, H-15), 1.05 (3H, d, J = 7, H-12′), 1.16 (1H, m, H-8β), 1.22 (3H, s, H-13), 1.31 (3H, s, H-14), 1.41 (3H, s, H-12), 1.44 (1H, m, H-10′), 1.62 (1H, dd, J = 9.5, 9.5 Hz, H-5), 1.63 (1H, m, H-11′), 1.79 (1H, m, H-3α), 1.91 (1H, m, H-8α), 1.94 (1H, m, H-9α), 2.04 (1H, m, H-2α), 2.06 (1H, ddd, J = 6.5, 6.5, 6.5 Hz, H-9β), 2.16 (1H, m, H-3β), 2.19 (1H, m, H-2β), 2.39 (1H, ddd, J = 9.5, 9.5, 3.5 Hz, H-1), 2.68 (1H, ddd, J = 12.5, 12.5, 4 Hz, H-10′), 3.80 (1H, dd, J = 12.5, 4 Hz, H-9′), 10.53 (1H, s, H-7′), 10.56 (1H, s, H-8′).
- *Macrocarpal B (**5**)*: Pale yellow powder, ESI-MS *m/z*: 471 $[M-H]^-$. ^1H NMR (500 MHz, pyridine-d_5) δ: 0.65 (1H, ddd, J = 10, 10, 6 Hz, H-7), 0.78 (1H, dd, J = 10, 10 Hz, H-6), 1.05 (3H, dd, J = 7 Hz, H-13′), 1.08 (3H, d, J = 7 Hz, H-12′), 1.12 (1H, m, H-8β), 1.16 (3H, s, H-13), 1.30 (3H, s, H-14), 1.38 (1H, m, H-3α), 1.41 (3H, s, H-15), 1.42 (3H, s, H-12), 1.50 (1H, dd, J = 10, 10 Hz, H-5), 1.60 (2H, m, H-10, 11′), 1.89 (1H, m, H-3β), 1.92 (2H, m, H-8α, 9α), 1.96 (1H, m, H-2α), 2.04 (1H, ddd, J = 6.5, 6.5, 6.5, H-9β), 2.18 (1H, m, H-2β), 2.43 (1H, ddd, J = 10, 10, 6 Hz, H-1), 2.79 (1H, ddd, J = 13, 13, 9 Hz, H-10′), 3.70 (1H, dd, J = 13, 9 Hz, H-9′), 10.54 (1H, s, H-7′), 10.57 (1H, s, H-8′).
- *Macrocarpal D (**6**)*: Pale yellow powder, ESI-MS *m/z*: 471 $[M-H]^-$. ^1H NMR (500 MHz, pyridine-d_5) δ: 1.00 (3H, d, J = 6.9 Hz, H-14), 1.04 (3H, d, J = 6.5 Hz, H-12′), 1.10 (3H, d, J = 6.5 Hz, H-13′), 1.21 (3H, s, H-15), 1.41 (1H, m, H-9), 1.44 (1H, m, H-8), 1.47 (3H, s, H-12), 1.48 (1H, m, H-3), 1.54 (3H, s, H-13), 1.57 (1H, m, H-2), 1.59 (1H, m, H-10′), 1.65 (1H, m, H-11′), 1.75 (1H, m, H-2), 1.84 (1H, ddd, J = 17, 12, 4.1 Hz, H-8), 2.07 (1H, m, H-10), 2.16 (1H, m, H-9), 2.49 (1H, m, H-7), 2.52 (1H, m, H-3), 2.76 (1H, ddd, J = 17, 12, 4.1 Hz, H-10′), 3.01 (1H, m, H-1), 4.02 (1H, dd, J = 9, 3.6 Hz, H-9′), 10.53 (1H, s, H-7′), 10.56 (1H, s, H-8′).
- *Macrocarpal E (**7**)*: Pale yellow powder, ESI-MS *m/z*: 471 $[M-H]^-$. ^1H NMR (500 MHz, pyridine-d_5) δ: 0.91 (3H, d, J = 6.4 Hz, H-12′), 0.97 (3H, d, J = 6.4 Hz, H-13′), 1.11 (3H, d, J = 7.5 Hz, H-15), 1.36 (3H, s, H-14), 1.45 (6H, s, H-12, 13), 1.48 (3H, s, H-11′), 1.53 (1H, m, H-3), 1.54 (1H, m, H-10′), 1.65 (1H, m, H-2), 1.67 (1H, m, H-3), 1.90 (1H, m, H-8), 1.93 (1H, m, H-1), 1.95 (1H, m, H-2), 1.99 (1H, m, H-9), 2.02 (1H, m, H-8), 2.10 (1H, m, H-9), 2.40 (1H, m, H-7), 2.50 (1H, m, H-4), 2.71 (1H, dd, J = 10, 10 Hz, H-10′), 3.89 (1H, m, H-9′), 5.87 (1H, d, J = 3 Hz, H-6), 10.55 (1H, s, H-7′), 10.56 (1H, s, H-8′).
- *Macrocarpal C (**8**)*: Pale yellow powder, ESI-MS *m/z*: 453 $[M-H]^-$. ^1H NMR (500 MHz, pyridine-d_5) δ: 0.70 (1H, m, H-7), 0.75 (1H, dd, J = 10, 10 Hz, H-6), 0.94 (3H, d, J = 6.5 Hz, H-12′), 0.99 (3H, d, J = 6.5 Hz, H-13′), 1.08 (3H, s, H-15), 1.10 (1H, m, H-8β), 1.18 (3H, s, H-13), 1.22 (3H, s, H-12), 1.37 (1H, m, H-10′), 1.57 (1H, m, H-11′), 1.60 (1H, m, H-3β), 1.63 (1H, dd, J = 10, 10 Hz, H-5), 1.81 (1H, m, H-2α), 2.00 (1H, m, H-2β), 2.04 (1H, m, H-8α), 2.09 (1H, m, H-9α), 2.45 (1H, m, H-1), 2.48 (1H, m, H-9β), 2.56 (1H, m, H-3α), 2.65 (1H, ddd, J = 12.5, 12.5, 3.5 Hz, H-10′), 3.66 (1H, dd, J = 12.5, 4, H-9′), 4.82 (1H, s, H-14), 4.94 (1H, s, H-14), 10.53 (1H, s, H-7′), 10.56 (1H, s, H-8′).

2.8. Antioxidative assays

The antioxidative activities were estimated using reported methods or these modified in part, inhibitions of the linoleic acid peroxidation assay [ferric thiocyanate and thiobarbituric acid (TBA) methods (Azuma et al., 1999; Mitsuda, Yasumoto, & Iwaki, 1966; Osawa & Namiki, 1985; Tanaka, Kusano, & Kouno, 1998)] and of the superoxide anion radical generation assay [nitro blue tetrazolium (NBT) method (Fukuda & Nakata, 1999; Oberley & Spitz, 1984; Okamura et al., 1993)], and free radical scavenging assay [1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Hatano, Takagi, Ito, & Yoshida, 1997)]. All experiments were carried out in triplicate and repeated at least three times.

For inhibition of linoleic acid peroxidation, the reaction mixture was composed of linoleic acid in ethanol (0.5 ml), the sample solution (0.1 ml), 0.1 mol/l phosphate buffer (pH 7.0) (0.5 ml) and water (1.0 ml). The peroxidation was initiated by the addition of 0.5 mol/l 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) (0.01 ml) [or 0.02 mol/l 2,2′-azobis(2,4-dimethylvaler-

onitrile) (AMVN) (0.05 ml)]. The mixture was incubated at 37 °C for 20 h in a brown tube. Following the incubation, the degree of oxidation was measured according to the ferric thiocyanate and TBA methods.

Inhibition of superoxide anion radical generation (NBT method) was estimated by the xanthine-xanthine oxidase (XOD) system, used as the source of the superoxide anion radical. The sample solution (0.1 ml) was added to the mixture (1 ml), consisting of 0.4 mmol/l xanthine and 0.24 mmol/l NBT (final concentration of NBT: 0.059 mmol/l) in phosphate buffer (pH 8.0). Xanthine oxidase (from butter milk, 0.049 unit/ml) (1.0 ml), diluted in 0.1 mol/l phosphate buffer (pH 8.0), was added, followed by incubation in a shaking water bath at 37 °C for 20 min, and then the coloration of NBT was measured at 560 nm. The reductive activity on the NBT was expressed as IC_{50}, the concentration of the test sample required to give a 50% reduction in the absorbance from that of 0.059 mmol/l NBT.

Scavenging of free radical capacity (DPPH method) of the samples was tested as bleaching of the stable DPPH. A solution (4 ml) of a test sample in MeOH was added to a solution (1 ml) of DPPH in MeOH (final concentration of DPPH: 0.2 mmol/l). After mixing for 10 s, the solution was left to stand for 30 min, and the absorbance of the resulting solution at 520 nm was measured. The scavenging activity on the DPPH radical was expressed as EC_{50}, the concentration of the test sample required to give a 50% reduction in the absorbance from that of 0.2 mmol/l DPPH in MeOH.

3. Results and discussion

3.1. Isolation and identification of constituents

The three-dimensional HPLC chromatogram of the eucalyptus product showed the coexistence of several compounds with absorption maxima at ca. 250–300 nm, as shown in Fig. 1. Accordingly, the HPLC analysis at 270 nm was carried out, and the chromatogram revealed at least eight peaks as major constituents of the sample [Fig. 2(a)]. For the purpose of isolation and purification of the detected compounds, the sample was successively extracted with *n*-hexane, ethyl acetate and *n*-butanol. The chromatograms of each extract are depicted in Fig. 2(b)–(e). Repeated preparative chromatographies, under the conditions described above for each extract, gave eight compounds (**1**–**8**).

Compounds **1** and **2** were identified as the known gallic acid (**1**) and ellagic acid (**2**), which are typical low-molecular-weight polyphenolics, widely distributed in many higher plants (Okuda, Yoshida, & Hatano, 1995), by direct comparison with authentic specimens. These compounds exist as free-forms in many plants, or as secondary plant metabolites or degradation products from gallotannins or ellagitannins, which are among the hydrolyzable tannins (Okuda, Yoshida, & Hatano, 1995,). Additionally, the isolated compounds are found to have antioxidative properties (Hatano et al., 1989; Okuda et al., 1995; Osawa, 1988; Osawa, Ide, Su, & Namiki, 1987; Yoshida et al., 1989), and the corresponding compounds originating from *Rhus javanica* and *Quercus infectoria* in **1** and *E. globulus*, *Trapa japonica*, *R. javanica*, *Terminalia chebula* in **2** are included in the List of Existing Food Additives as natural antioxidants in Japan [Notification No. 120 (16 April 1996), Ministry of Health and Welfare, Japan].

The NMR spectra of compounds **3**–**8** were found to possess an isobutyl side chain, a phloroglucinol unit bearing two aldehydes and a methane carbon bearing an isobutyl side chain, and hence these were presumed to be sesquiterpene-isophenyl phloroglucinol-coupled compounds. Based on each detailed structural analysis, their structures were identified as the known eucalyptone (**3**), macrocarpals A (**4**), B (**5**), D (**6**), E (**7**) and C (**8**), respectively, by comparisons of their physicochemical data with those reported in the literature (Murata, Yamakoshi, Homma, Aida, Hori, & Ohashi, 1990; Nishizawa, Emura, Kan, Yamada, Ogawa, & Hamanaka, 1992; Osawa, Yasuda, Morita, Takeya, & Itokawa, 1994; Yamakoshi, Murata, Shimizu, & Homma, 1992). The isolation and characterization of the phloroglucinol-sesquiterpene-coupled constituents from *E. globulus* and *E. macrocarpa* have already been presented, and their antibacterial activities against bacteria, such as *Staphylococcus aureus*, *Bacillus subtilis* (Murata et al., 1990; Nishizawa et al.; Osawa et al., 1994; Yamakoshi et al.) and oral pathogenic microorganisms, such as *Streptococcus mutans* and *S. sobrinus* (Osawa, 1998; Osawa, Saeki, Yasuda, Morita, Takeya, & Itokawa, 1998), and glucosyltransferase (Osawa et al., 1998), aldose reductase (Murata, Yamakoshi, Homma, Arai, & Nakamura, 1992) and HIV-Rtase (Nishizawa et al.) have been also reported.

The structures of the isolated and identified compounds from the eucalyptus product are depicted in Fig. 3.

3.2. GC/MS analysis

It is well-known that eucalyptus plants also produce terpenoidal hydrocarbons, and the essential oils, the so-called eucalyptus oils, are grouped as medicinal, industrial and perfumery types, depending on their chemical composition (Chiba, 1998; Nishimura & Calvin, 1979). The mono- and sesquiterpenes in the eucalyptus oil have been reported (Nishimura & Calvin) and the major components definitely include the monoterpene, 1,8-cineole. Accordingly, to analyze their compositions in the eucalyptus product, a GC/MS analysis of this product was attempted.

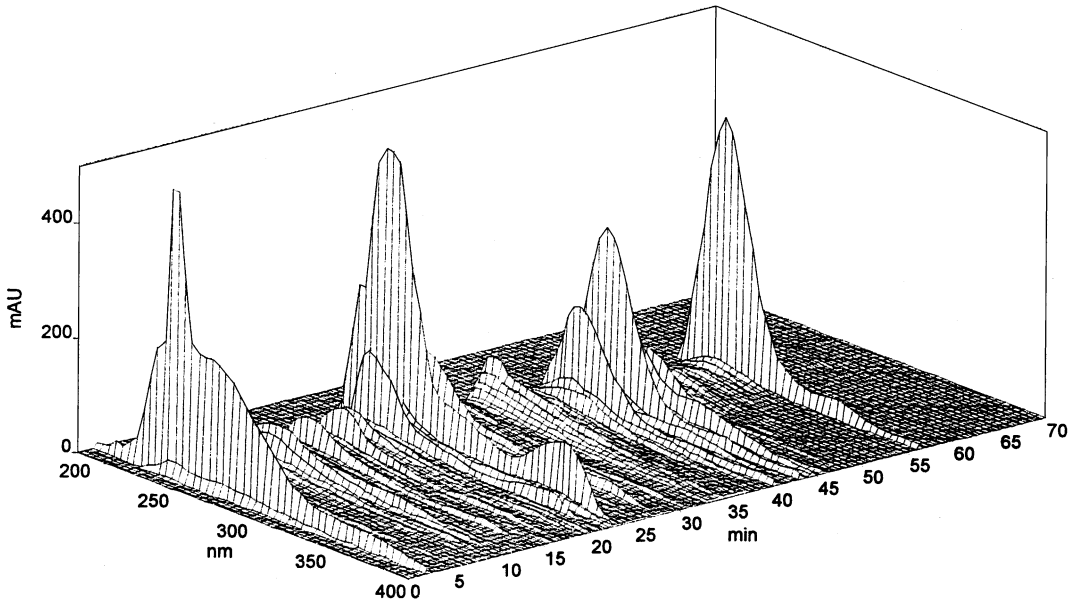

Fig. 1. Three-dimensional HPLC chromatogram of the eucalyptus product.

The GC/MS total ion chromatogram of the eucalyptus product was obtained under the conditions described above, as shown Fig. 4. The 1,8-cineole (**9**) was identified by GC co-injection of the standard and direct comparison of the MS data with that of the standard. The others were presumed from the comparisons of the fragment pattern of their MS spectra with those reported in the literature (Nishimura & Calvin, 1979). Other major detected peaks, were those of α-terpineol acetate (**10**), aromadendrene (**11**), globulol (**12**) and sesquiterpene alcohol (**13**), respectively. The largest major peak was that of **11**. Compounds **11** and **12** are sesquiterpene moieties of macrocarpals isolated from this eucalyptus product, and the detected **13** is also presumably one of them. On the other hand, peak **10** was detected as a peak slightly smaller than the others. Actually, the content of **10** in this product was determined, by the absolute calibration method, from the peak areas of the GC/MS (SIM) chromatogram, and the result was 0.05 mg/g product weight.

3.3. Quantitative analysis

The contents of the isolated compounds were quantified by an absolute calibration method from the peak areas of the HPLC chromatogram. Table 2 shows their contents in the eucalyptus product. Compounds **1** and **2** exhibited contents of 1.68 and 1.32 mg/g of the eucalyptus product weight, respectively. The phloroglucinol derivatives had contents in the range of 0.86–2.36 mg/g. The primary compound was **8** with 2.36 mg/g.

On the other hand, the amount of total polyphenolics in the eucalyptus product was determined by the Folin–Ciocalteu method, based on gallic acid. The content of

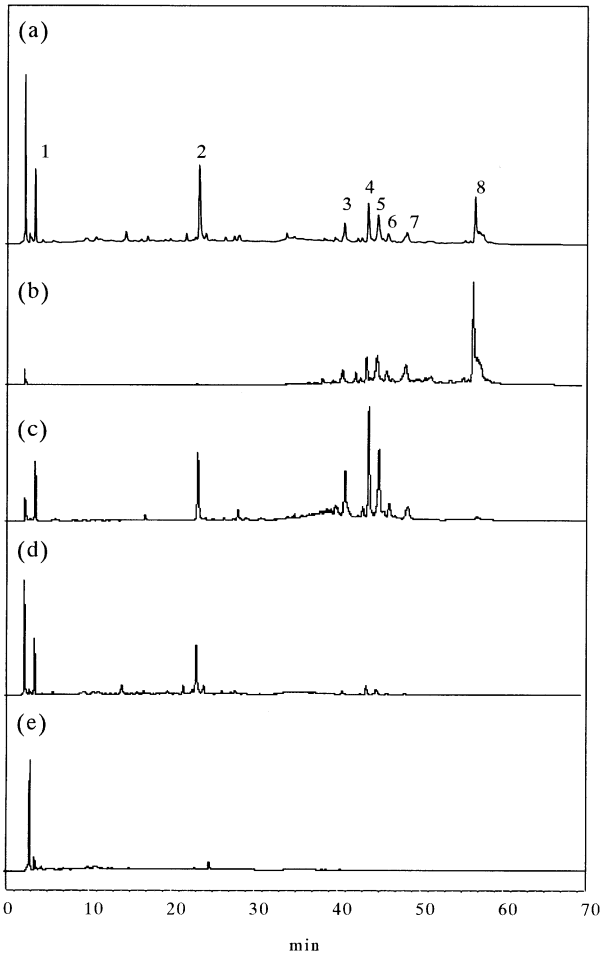

Fig. 2. HPLC chromatograms of the eucalyptus product and its extracts. (a) eucalyptus product, (b) *n*-hexane extract, (c) ethyl acetate extract, (d) *n*-butanol extract, (e) residure. Peaks: 1, gallic acid; 2, ellagic acid; 3, eucalyptone; 4, macrocarpal A; 5, macrocarpal B; 6, macrocarpal D; 7, macrocarpal E; 8, macrocarpal C.

the total polyphenolics was 11.9 mg/g (gallic acid equivalents). This fact indicated that the total polyphenolic content roughly corresponded to that of the eight isolated compounds' weight (12.1 mg/g).

3.4. Antioxidative activity

The antioxidative activity of each extract and isolated compound were examined by several model systems,

Table 1
Contents, inhibition of superoxide anion radical generation and scavenging of 1,1-diphenyl-2-picrylhydrazyl radical of extracts from eucalyptus product

	Weight (g)[a]	NBT IC_{50} (mg/ml)[b]	DPPH EC_{50} (mg/ml)[c]
Eucalyptus sample		5.51	0.41
n-Hexane extract	0.63	2.07	0.43
Ethyl acetate extract	2.52	0.66	0.11
n-Butanol extract	13.7	1.51	0.28
Residue	1.51	2.74	0.34

[a] Amount of extracted from 50 g of eucalyptus product.
[b] Concentration required for a 50% reduction in absorbance of the NBT at 560 nm.
[c] Concentration required for a 50% reduction in absorbance of DPPH radical at 520 nm.

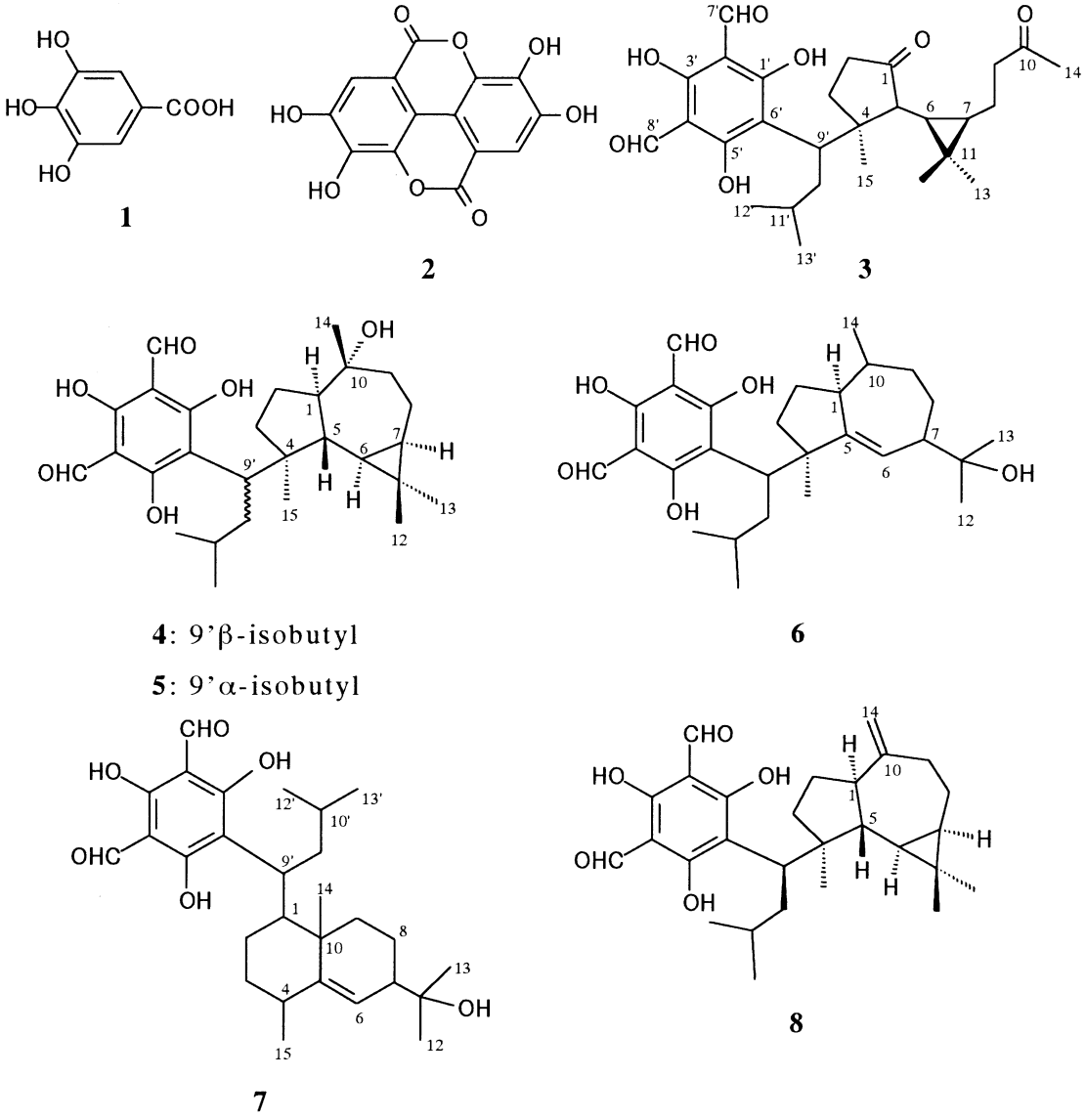

Fig. 3. Chemical structures of isolated compounds from the eucalyptus product.

using the ferric thiocyanate, TBA, NBT and DPPH methods.

The inhibitions of activities against lipid peroxidation in linoleic acid, caused by AAPH as water-soluble radical initiator or AMVN as a lipophilic radical initiator, were evaluated by measuring the concentration of the TBA-reactive substances and ferric thiocyanate. The values obtained without antioxidants were taken for 100% lipid peroxidation. As shown in Fig. 5, compared to that of each extract, the ethyl acetate extract was inhibited to some extent by the lipid peroxidation, caused by both the lipid-soluble and water-soluble radical generators, successively, followed by the *n*-butanol and *n*-hexane extracts, successively. Similarly, the inhibition of activities against superoxide anion radical generation in the xanthine–XOD system was evaluated by measuring the concentration of diformazan-reduced NBT, and the scavenging of the free radicals was

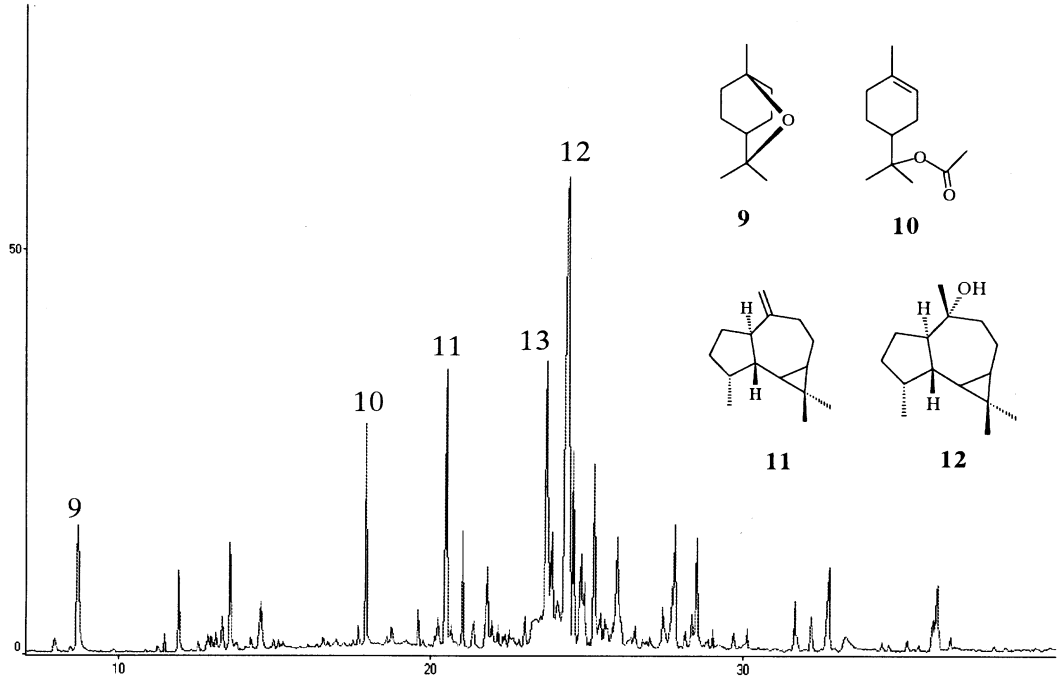

Fig. 4. GC/MS total ion chromatogram of *n*-hexane extract from the eucalyptus product. Peaks: 9, 1,8-cineole; 10, α-terpineol acetate; 11, aromandendrene; 12, globulol; 13, sesquiterpene alcohol.

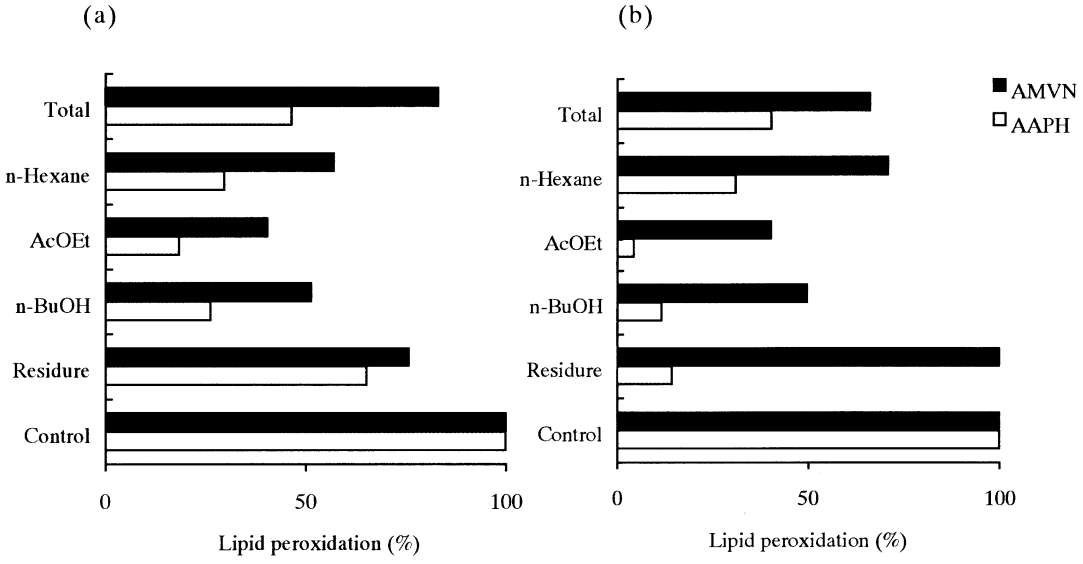

Fig. 5. Inhibition activity against lipid peroxidation of linoleic acid caused by water-soluble (AAPH) and lipid-soluble (AMVN) radical initiators of extraction from the eucalyptus product. The values obtained without antioxidants were used for 100% lipid peroxidation. Each sample was added to the solution at a concentration of 10 mg/ml: (a) ferric thiocyanate method; (b) TBA method.

estimated by bleaching of the stable DPPH radical. Compared to the inhibition activities (IC_{50} in NBT and EC_{50} in DPPH values) of each extract from the eucalyptus product, as shown in Table 1, the ethyl acetate extract showed the most potent activity (IC_{50} and EC_{50} values, 0.66 and 0.11 mg/ml). The *n*-butanol extract was secondary with amounts of 1.51 and 0.28 mg/ml, followed by the *n*-hexane extract. Considering the amounts extracted from this product, the *n*-butanol extract had the largest quantity among them, as shown in Table 1 (13.7 g). Therefore, this extract might contain the majority of the antioxidative activity. Compounds co-detected in the HPLC chromatograms of the ethyl acetate and *n*-butanol extracts that had impressive potencies in all the tested methods were **1** and **2**. From these results, it was presumed that the body of antioxidative activity in this sample was due to their typical polyphenolics. Accordingly, the isolated compounds were examined by the same tests. Compared to the eight compounds isolated, as shown in Fig. 6 and Table 2, **1** and **2** strongly inhibited lipid peroxidation and the activities against superoxide anion radical generation, and scavenged the DPPH stable radical, as would be predicted. They were more potent than or comparable to that of BHA and BHT. On the other hand, the phloroglucinol derivatives, such as the macrocarpals, rarely showed lipid peroxidation inhibition, and **10**, which is known as a major component in eucalyptus oil, likewise showed no appreciable effects, even at high concentrations.

In summary, the chemical components of the eucalyptus leaf extract, as a natural food additive, were

Table 2
Contents, inhibition of superoxide anion radical generation and scavenging of 1,1-diphenyl-2-picrylhydrazyl radical of isolated compounds from eucalyptus product

	Content (mg/g)[a]	NBT EC_{50} (μmol/l)[b]	DPPH EC_{50} (μmol/l)[c]
Gallic acid (1)	1.68	225	18.3
Ellagic acid (2)	1.32	259	13.6
Eucalyptone (3)	1.20	>1000	>100
Macrocarpal A (4)	1.80	>1000	>100
Macrocarpal B (5)	1.96	>1000	>100
Macrocarpal D (6)	0.86	>1000	>100
Macrocarpal E (7)	0.92	>1000	>100
Macrocarpal C (8)	2.36	>1000	>100
1,8-Cineole	0.05	>1000	>100
BHA		>1000	37.8
BHT		>1000	84.2

[a] Amount in eucalyptus product weight.
[b] Concentration required for a 50% reduction in absorbance of the NBT at 560 nm.
[c] Concentration required for a 50% reduction in absorbance of DPPH radical at 520 nm.

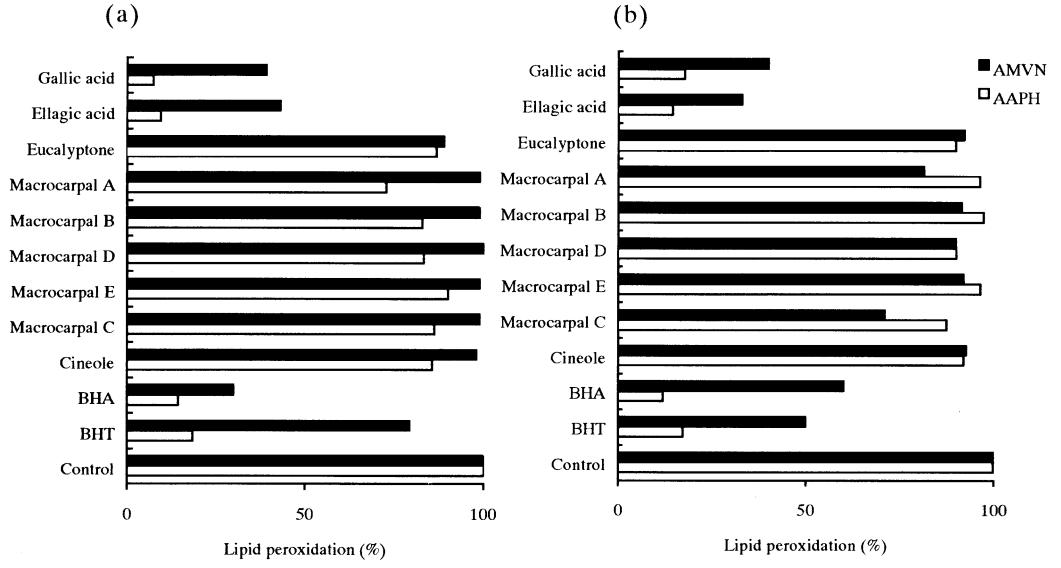

Fig. 6. Inhibition activity against lipid peroxidation of linoleic acid caused by water-soluble (AAPH) and lipid-soluble (AMVN) radical initiators of extraction from the eucalyptus product. The values obtained without antioxidants were used for 100% lipid peroxidation. Each sample was added to the solution at a concentration of 1.0 mg/ml: (a) ferric thiocyanate method; (b) TBA method.

isolated and identified. First, the structures of eight major compounds, namely **1** and **2** as typical low-molecular-weight polyphenolics, and **3**–**8** as phloroglucinol derivatives, isolated from this product were elucidated by spectroscopic methods. The primary compound was **8**. Next, the antioxidative activities of the compounds isolated from the eucalyptus product were estimated by several methods, and it is suggested that the majority of the antioxidant activity in this product is due to **1** and **2**. On the other hand, the low-polar compounds were analyzed by GC/MS, and monoterpenes, such as **9** and sesquiterpenes such as **12**, were detected. In the eucalyptus product, it was observed that the content of **9**, a major component of the eucalyptus oil, was low, and its activity as an antioxidant was negligible.

In this present study, only one obtained product, was studied. Therefore, the fluctuation in content levels of the included constituents of the eucalyptus product, based on differences in plant material origin, or preparation methods, might be predictable. For example, if the products were manufactured using a less polar solvent than that of the product studied, it might be predictable that low-polar compounds, such as **9**, could be detected in larger quantities. Moreover, it has been reported that the leaves of the eucalyptus plants contain many hydrolyzable tannins, with significant antioxidative activities (Okamura et al., 1993; Okuda et al., 1995; Sugimoto, Nakagawa, & Hayashi, 2000). Therefore, **1** and **2**, antioxidants isolated from the eucalyptus product in this study, might be degradation products from the gallotannins or ellagitannins, which are types of hydrolyzable tannins.

4. Conclusions

1, **2** and related polyphenolics, and the phloroglucinol derivatives, such as **3**–**8**, monoterpenes, such as **9**, and the sesquiterpenes, such as **12**, were enumerated as major constituents of the eucalyptus product studied, and the major activity was anticipated to be due to **1**, **2** and their related polyphenolics.

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References

Azuma, K., Nakayama, M., Koshioka, M., Ippoushi, K., Yamaguchi, Y., Kohata, K., Yamauchi, Y., Ito, H., & Higashio, H. (1999). Phenolic antioxidants from the leaves of *Corchorus olitorius* L. *Journal of Agricultural and Food Chemistry*, *47*(10), 3963–3966.

Chiba, K. (1998). Electrochemical characterization of naturally occurring phenolic antioxidants. *Nippon Yuka Gakkaishi (Journal of Japan Oil Chemist's Society)*, *47*(8), 743–751.

Fukuda, Y., & Nakata, S. (1999). Effect of roasting temperature in sliced almonds and sesame seeds on the antioxidative activities. *Nippon Shokuhin Kagaku Kogakkaishi (Journal of the Japanese Society for Food Science and Technology)*, *46*(12), 786–791.

Goda, Y. (1994). Various plants used as the source of natural food additives in Japan. *Shokubutsu Saibo Kogaku (Plant Cell Technology)*, *6*(2), 98–108.

Hatano, T., Edamatsu, R., Hiramatsu, M., Mori, A., Fujita, Y., Yasuhara, T., Yoshida, T., & Okuda, T. (1989). Effects of interaction of tannins with co-existing substances. VI. Effects of tannins and related polyphenols on superoxide anion radical, and on 1,1-diphenyl-2-picrylhydrazyl radical. *Chemical and Pharmaceutical Bulletin*, *37*(8), 2016–2021.

Hatano, T., Takagi, M., Ito, H., & Yoshida, T. (1997). Phenolic constituents of liquorice. VII. A new calcone with a potent radical scavenging activity and accompanying phenolics. *Chemical and Pharmaceutical Bulletin*, *45*(9), 1485–1492.

Julkunen-Tiitto, R. (1985). Phenolic constituents in the leaves of northern willows. *Journal of Agricultural and Food Chemistry*, *33*(2), 213–217.

Mitsuda, H., Yasumoto, K., & Iwami, K. (1966). Antioxidative action of indole compounds during the autoxidation of linoleic acid. *Eiyo To Shokuryo (Journal of Japanese Society of Food and Nutrition)*, *19*(3), 210–214.

Murata, M., Yamakoshi, Y., Homma, S., Aida, K., Hori, K., & Ohashi, Y. (1990). Macrocarpal A, a novel antibacterial compound from *Eucalyptus globulus*. *Agricultural and Biological Chemistry*, *54*(12), 3221–3226.

Murata, M., Yamakoshi, Y., Homma, S., Arai, K., & Nakamura, Y. (1992). Macrocarpals, antibacterial compounds from *Eucalyptus*, inhibit aldose reductase. *Bioscience, Biotechnology and Biochemistry*, *56*(12), 2062–2063.

Nishimura, H., & Calvin, M. (1979). Essential oil of *Eucalyptus globulus* in California. *Journal of Agricultural and Food Chemistry*, *27*(2), 432–435.

Nishizawa, M., Emura, M., Kan, Y., Yamada, H., Ogawa, K., & Hamanaka, N. (1992). Macrocarpals: HIV-RTase inhibitors of *Eucalyptus globulus*. *Tetrahedron Letters*, *33*(21), 2983–2986.

Oberley, L. W., & Spitz, D. R. (1984). Assay of superoxide dismutase activity in tumor tissue. *Methods in Enzymology*, *105*, 457–464.

Okamura, H., Mimura, A., Yakou, Y., Niwano, M., & Takahashi, Y. (1993). Antioxidant activity of tannins and flavonoids in *Eucalyptus rostrata*. *Phytochemistry*, *33*(3), 557–561.

Okuda, T., Yoshida, T., & Hatano, T. (1992). Antioxidant effects of tannins and related polyphenols. In M.-T. Huang, C.-T. Ho, & C. Y. Lee (Eds.), *Phenolic compounds in food and their effects on health II* (pp. 87–97). Washington, DC: American Chemical Society.

Okuda, T., Yoshida, T., & Hatano, T. (1995). Hydrolyzable tannins and related polyphenols. *Progress in the Chemistry of Organic Natural Products*, *66*, 1–102.

Osawa, K., Yasuda, H., Morita, H., Takeya, K., & Itokawa, H. (1994). Eucalyptone from *Eucalyptus globulus*. *Phytochemistry*, *40*(1), 183–184.

Osawa, K. (1998). Preventive effect of eucalyptus leaf extract on dental caries and periodontitis. *Bio Industry*, *15*(11), 5–13.

Osawa, K., Saeki, T., Yasuda, H., Morita, H., Takeya, K., & Itokawa, H. (1998). Antibacterial activity of *Eucalyptus globulus* on cariogenic bacteria and its inhibitory effect on glucosyltransferase. *Natural Medicines (Tokyo)*, *52*(1), 32–37.

Osawa, T., & Namiki, M. (1985). Natrural antioxidants isolated from Eucalyptus leaf waxes. *Journal of Agricultural and Food Chemistry*, *33*(5), 777–780.

Osawa, T., Ide, A., Su, J.-D., & Namiki, M. (1987). Inhibition of lipid peroxidation by ellagic acid. *Journal of Agricultural and Food Chemistry*, *35*(5), 808–812.

Osawa, T. (1988). Natural antioxidants. *Nippon Nogeikagaku Kaishi*, *62*(2), 166–169.

Sugimoto, K., Nakagawa, K., & Hayashi, S. (2000). Physiological function and application of eucalyptus leaf extract. *Shokuhin To Kaihatsu (Up-to-date Foodprocessing)*, *35*(6), 15–18.

Tanaka, T., Kusano, R., & Kouno, I. (1998). Synthesis and antioxidant activity of novel amphiphathic derivatives of tea polyphenol. *Bioorganic and Medicinal Chemistry Letters*, *8*, 1801–1806.

Yamakoshi, Y., Murata, M., Shimizu, A., & Homma, S. (1992). Isolation and characterization of macrocarpals B-G antibacterial compounds from *Eucalyptus macrocarpa*. *Bioscience, Biotechnology and Biochemistry*, *56*(10), 1476–1570.

Yoshida, T., Mori, K., Hatano, T., Okumura, T., Uehara, I., Komagoe, K., Fujita, Y., & Okuda, T. (1989). Studies on inhibition mechanism of antioxidation by tannins and flavonoids. V. Radical-scavenging effects of tannins and related polyphenols on 1,1-diphenyl-2-picrylhydrazyl radical. *Chemical and Pharmaceutical Bulletin*, *37*(7), 1919–1921.

Yoshihira, K. (1996). The 3rd Japan Food Chemistry Seminar. Concerning the revision of the food sanitation law and natural additives. *Foods and food Ingredients Journal of Japan*, *168*, 113–128.