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A Novel Type of Antioxidant Isolated from Leaf Wax of *Eucalyptus* leaves

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As a search for natural antioxidants from plant materials, strong antioxidative activity was observed in leaf waxes extracted from *Eucalyptus* species. A novel type of antioxidant was isolated from the leaf wax of *Eucalyptus globulus* and identified as *n*-tritriacontan-16,18-dione. Antioxidative activities were determined by different methods; a thiocyanate method, a thiobarbituric acid method, a total carbonyl value method and a weighing test. The antioxidant showed remarkable antioxidative activity in a water/alcohol system and was more effective than α-tocopherol and BHA; however, it has no antioxidative activity in an oil system.

Hundreds of materials, both synthetic and of natural origin, have been developed as antioxidants for food preservation,[1] but only *tert*-butyl-4-hydroxyanisol (BHA) and *tert*-butyl-4-hydroxytoluene (BHT) as synthetic antioxidants and tocopherols as natural ones are practically used. However, the most widely used antioxidants, BHA and BHT, are suspected of causing liver damage. Moreover, synthetic chemicals used as food additives tend to be eliminated. On the other hand, tocopherols are widely used as safe natural antioxidants, but they are not so effective as synthetic antioxidants and the manufacturing cost is high.

These circumstances stimulated us to isolate a new antioxidant from natural sources, especially from plant materials. This report is concerned with the isolation, identification and characterization of a new antioxidant in the leaf wax extracted from *Eucalyptus* leaves, especially of *E. globulus*. *Eucalyptus* leaves contain a large amount of essential oils and they are stable in the tissues, so it seems probable that leaf waxes have some protective systems preventing oxidative degradation of oils not only by physical mechanisms but also by chemical factors. The essential oils in *Eucalyptus* species are important for medicinal, industrial and perfumary uses. Furthermore, it has recently been suggested that *Eucalyptus* oil can be used as an alternate source of hydrocarbon fuel.[2]

MATERIALS AND METHODS

Extraction of leaf wax. Leaves of thirteen species of *Eucalyptus* were collected from the Botanical Garden in Matsudo City, Chiba, Japan, and those of other four species (*E. dives*, *E. polybractea*, *E. pulverulenta* and *E. citiodora*) were kindly furnished by Dr. H. Nishimura of the Department of Agricultural Chemistry, Hokkaido University, Sapporo, Japan. Leaf waxes were extracted twice with chloroform, filtered and concentrated *in vacuo*.

Purification of an antioxidative substance (S-1). Five grams of leas wax obtained from *E. globulus* leaves (500 grams, *fresh* weight) was charged *on a silica gel column* (Wako Gel C-100, Wako Chemicals Co. Ltd.) with *n*-hexane-ethyl acetate as the solvent system. The fractions eluted with *n*-hexane and *n*-hexane-ethyl acetate (9: 1 v/v) exhibited a very strong antioxidative activity with the thiocyanate method, and were combined and subjected to preparative TLC (Wako Gel B-5 FM, 0.5 mm thickness) using *n*-hexane-ethyl acetate (3: 1 v/v) as the solvent system and active fractions at *Rf* 0.80 and 0.68 were scraped off and eluted with chloroform. Recrystallization of the *Rf* 0.80 fraction (main product) gave pure S-1 in a crystalline form (mp 67～68°C, yield; 10% from leaf wax). A compound (S-1) such as a β-diketone was separated as the copper complex by the alternate method and regenerated using aqueous hydrochloric acid,[3] followed by purification by preparative TLC (yield; 5% from leaf wax).

Antioxidative assay

1. Thiocyanate method. Different amounts of samples dissolved in 100 μl of chloroform were put

into a solution of linoleic acid (0.13 ml), 99.5% ethanol (10 ml) and 0.2 M phosphate buffer (10 ml), and the total volume was adjusted to 25 ml with distilled water. Linoleic acid was purchased from Wako Chemicals Co. Ltd. and vacuum distilled. The mixed solution in a conical flask was kept in a constant temperature oven at 40°C. At intervals during incubation, the peroxide value was determined by the method of Mitsuda *et al.*[4] using thiocyanate as a coloring reagent and the absorbance at 500 nm of the colored solution was measured with a photoelectric colorimeter (ERMA).

2. Thiobarbituric acid (TBA) test. Mixtures of linoleic acid and the sample were prepared and incubated as described above. The TBA value was calculated from the absorbance at 532 nm following Ottolenghi's method.[5]

3. Total carbonyl value (TCV) test. Two different methods of incubation of linoleic acid and the sample for the TCV test were carried out.

1) Water/alcohol system; the same as that mentioned in the thiocyanate method.

2) Oil system; different amounts of the chloroform solution of the subject sample were placed in petri dishes. After chloroform was removed completely by a stream of nitrogen, one gram of linoleic acid was added and the mixture stood in a constant temperature oven at 40°C.

At intervals during incubation, the carbonyl value was determined by the 2,4-dinitrophenylhydrazine test according to the method of Kumazawa *et al.*[6]

4. Weighing method. The rate of oxidation of oil was determined by weighing the sample at intervals during incubation in a constant temperature oven following the method developed by Olcott and Einset.[7] Preparation of the sample was the same as described in the TCV test (oil system) and at definite intervals the petri dishes were removed from the oven, allowed to cool at room temperature for 30 min in a desiccator, weighed and replaced into the oven.

Instruments for structural elucidation. UV; Hitachi 200-10 spectrometer, IR; Jasco A-3; NMR; Jeol JNM-FX-100, mass spectrum; Jeol JNM-D-100 and high resolution mass spectrum; Jeol JMS-OISG.

Thin Layer chromatographic analysis. TLC was carried out on a fluorescent silica gel (Wako gel FM-plate, 0.25 mm thickness) with *n*-hexane–ethyl acetate (3 : 1 v/v). The spots were observed under UV light and the amount of S-1 was determined quantitatively using a Shimadzu chromatoscanner by calculation from absorbance at 280 nm.

RESULTS

Antioxidative activity of leaf wax extracted from Eucalyptus leaves

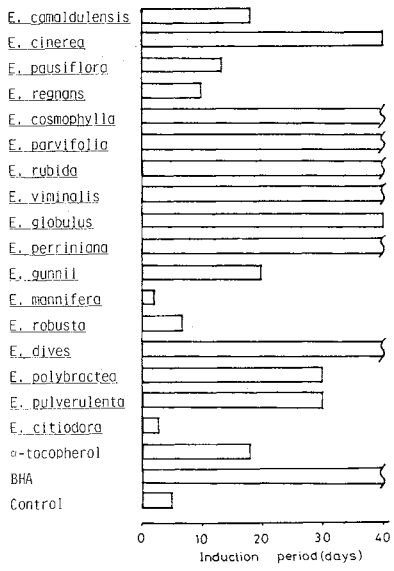


FIG. 1. Antioxidative Activity of Leaf Wax Extracted from *Eucalyptus* Leaves.

Antioxidative activity was assayed by the thiocyanate method. 1 mg of leaf wax, 100 μg of α-tocopherol and BHA were used for this assay.

The leaf waxes extracted from leaves of seventeen species of *Eucalyptus* were dissolved in chloroform (10%, w/v) and each 100 μl of the solutions was used for antioxidative assay by the thiocyanate method. As shown in Fig. 1, *E. regnans*, *E. manifera*, *E. robusta* and *E. citiodora* have no antioxidative activity, but the other thirteen *Eucalyptus* species have antioxidative activity, especially, it was prominent in the cases of *E. cosmophylla*, *E. parvifolia*, *E. rubida*, *E. viminalis*, *E. perriniana* and *E. dives* whose induction periods were more than forty days.

Thin layer chromatographic analysis of leaf wax extracted from E. globulus

Detailed investigation of the active components in the leaf wax extracts by use of TLC analysis was carried out on the leaf wax extracted from *E. globulus*, because leaves of *E. globulus* are easily obtainable in large amounts in Japan. As shown in Fig. 2, eleven spots were observed on TLC and each band of the preparative TLC corresponding to these spots was scraped off and extracted

with chloroform. Each 100 μg of the fractionated products was assayed by the thiocyanate method and a significant antioxidative activity was observed for the S–1 and S–2 fractions (induction periods of these fractions were more than 45 days). S–1 is the main product (about 10% yield from leaf wax), for which identifi-

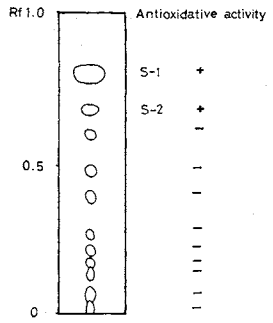
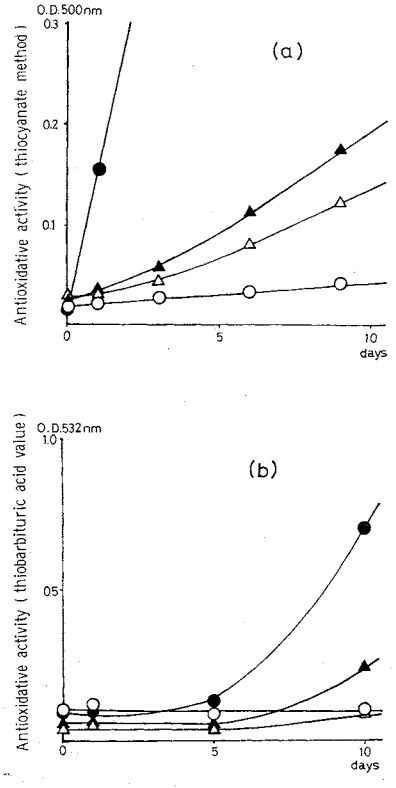


FIG. 2. TLC and Antioxidative Activity of Leaf Wax Extracted from *Eucalyptus globulus*.

TLC analysis was carried out using a Wako Gel FM Plate (0.25 mm) and *n*-hexane–ethyl acetate (3: 1) as the solvent system. Antioxidative activity of each fraction (100 μg) was assayed by the thiocyanate method.



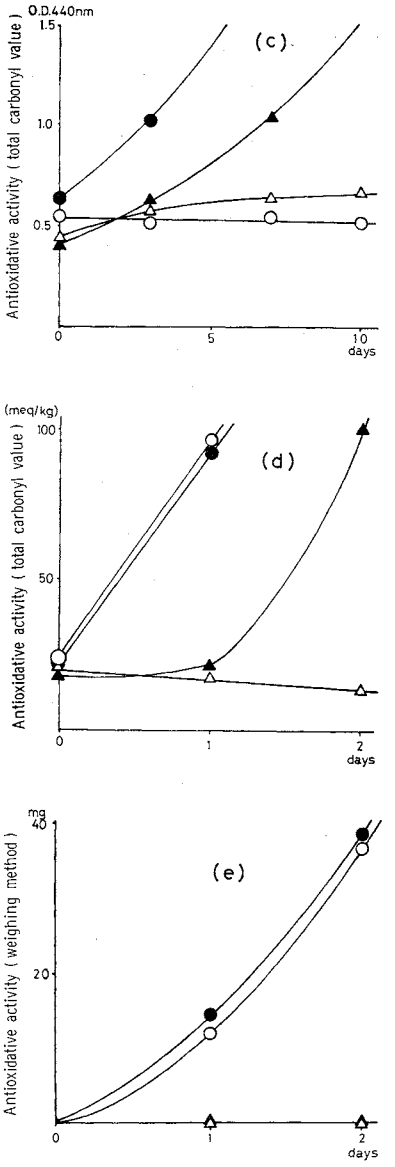


FIG. 3. Antioxidative Assay of S–1, α-Tocopherol and BHA.

100 μg of each sample was used for these assays. ●, control; ○, S–1; △, BHA and ▲, α-tocopherol. (a), thiocyanate method; (b), thiobarbituric acid test; (c), total carbonyl value test (water/alcohol system); (d), total carbonyl value test (oil system)

Total carbonyl value (meq/kg) =
(absorbance/0.854 × W) × 1000

W, weight of sample and oil; absorbance, O.D. at 440 nm; (e), weighing method.

cation of the chemical structure and further antioxidative assays using various methods were undertaken.

Antioxidative activities of S–1

The antioxidative activity of S–1 in a water/alcohol system was examined by the thiocyanate method as shown in Fig. 3-a. S–1 showed a marked antioxidative activity and it was more effective than α-tocopherol and BHA.

It was also shown that S–1 inhibited the production of malonaldehyde and carbonyl compounds during the oxidative degradation of linoleic acid. The results of the TBA test in the water/alcohol system are shown in Fig. 3-b, S–1 was shown to be as effective as BHA and more active than α-tocopherol. S–1 was also observed to inhibit the formation of carbonyl compounds by the TCV test in the water/alcohol system and it is indicated that S–1 is more active than α-tocopherol and BHA (Fig. 3-c). It was thus demonstrated that S–1 has a very strong antioxidative activity in the tests using the water/alcohol system. To determine the antioxidative activity in another system, the TCV test in an oil system was carried out. As shown in Fig. 3-d, S–1 did not show any antioxidative activity in this system. A confirmatory experiment on the activity in the oil system was carried out and the rate of oxidation of linoleic acid was measured in the oil system by the weighing method. The results shown in Fig. 3-e also indicated that S–1 was inactive in the oil system. These results suggest that S–1 has the antioxidative activity only with the presence of water in the system, though details of its antioxidative process are still unknown.

Quantitative determination of S–1 in Eucalyptus species

The amount of S–1 in the leaf waxes extracted from seventeen species of *Eucalyptus* leaves was determined (Fig. 4). The correlation between the concentration of S–1 and the antioxidative activity determined by the thiocyanate method was observed except for three *Eucalyptus* species; *E. camaldlensis, E. pauciflora* and *E. polybractae* (see also Fig. 1). However, in the leaf waxes of *E. parvifolia* and *E. rubida* the concentration of S–1 was not so high but the antioxidative activity was very strong as shown in Fig. 1. It is thus suggested that these five species may contain antioxidants other than S–1 and detailed examination is now in progress.

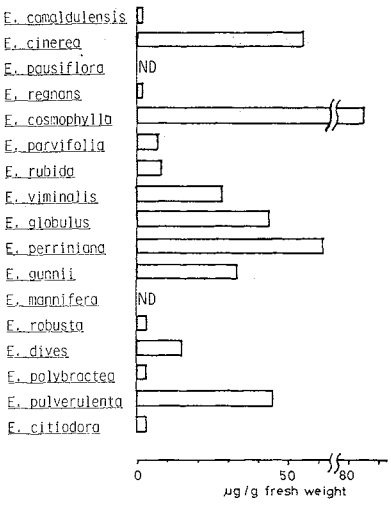


FIG. 4. Quantitative Determination of S–1 in *Eucalyptus* Species.

Structural analysis of S–1

The molecular formula of S–1 was confirmed by the high resolution mass spectrum which gave a molecular ion peak at *m/z* 492.4925 (492.4906; calcd. for $C_{33}H_{64}O_2$) and fragment peaks at *m/z* 477, 310, 297, 281 (M^+-$C_{15}H_{31}$) dan 239 (M^+-$C_{15}H_{31}COCH_2$). The ^{1}H-NMR spectrum in $CDCl_3$ showed a triplet at δ 0.9 ppm ($2\cdot CH_3$), multiplet at δ 1.3 ppm ($26\cdot CH_2$) and a triplet at δ 2.3 ppm ($2\cdot CO–C\underline{H}_2–CH_2$). S–1 showed keto-enol tautomerism and protons of the keto form were observed at δ 3.5 ppm (s, $CO–C\underline{H}_2–CO$) and that of the enol form was at δ 5.4 ppm (s, $CO–C\underline{H}=C(OH)–$) and their ratio was confirmed to be 1 : 6 by calculation from the integrated protons. The ^{13}C-NMR spectrum exhibited methyl carbons at δ 14.0 ppm (q), $–\underline{C}H_2–CH_3$ at δ 23.0 ppm (t), $–\underline{C}H_2–CH_2–CH_3$ at δ 25.0 ppm (t), $–(\underline{C}H_2)_{10}–CH_2–CH_2–CH_3$ at δ 30.0 ppm (m), $–CO–CH_2–CH_2–$

n-tritriacontan-16,18-dione

FIG. 5.

at δ 32.0 ppm (t) and $-CO-\underline{C}H_2-CH_2-$ at δ 38.0 ppm (t). The methylene carbon of the keto form was observed at δ 43.0 ppm (t) and carbonyl carbons at δ 194.0 ppm (s). Olefinic carbons of the enol form were observed at δ 99.0 ppm (d) and δ 115.0 ppm (s). S–1 has absorption at 2930 and 2850 cm^{-1} (ν_{CH}), 1610 cm^{-1} (C=O), 1465, 1450 and 1370 cm^{-1} (δ_{CH}) in the IR spectrum (KBr), and λ_{max} (chloroform) 274 nm (ε=8000) in the UV spectrum.

By these instrumental analyses and investigations in the literature, S–1 was identified as *n*-tritriacontan-16,18-dione which was first isolated as a component of leaf waxes extracted from *Eucalyptus* species by Horn *et al.*[3] and the chemical structure of S–1 is shown in Fig. 5.

DISCUSSION

Most synthetic and natural antioxidants have phenolic hydroxy groups in the structures, however, it is indicated that S–1 is a completely new type of antioxidant which belongs to the β-diketones. Because S–1 has the very strong antioxidative activity only in the water/alcohol system, it is suggested that S–1 may be active as the enol form in the presence of water. However, acetylacetone, which is the simplest β-diketone and whose ratio (keto form: enol form) is also 1: 6, has no antioxidative activity at all and this suggests that long hydrocarbon chain moieties may be essential for the activity. In order to get more information on the relationship between the antioxidative activity and structure, synthesis of β-diketone derivatives is being undertaken. The possibility of the presence of other antioxidant(s) in five *Eucalyptus* species and isolation of S–2 present in *E. globulus* are also under investigation.

The essential oils extracted from *Eucalyptus* leaves are widely added to foodstuffs, cosmetics and medicines and also Koala bears in Australia eat only *Eucalyptus* leaves. These facts support the safety of utilization of S–1 as a natural antioxidant. S–1 may be utilized for medical purposes to inhibit peroxidation of lipids in the living body. The present work has been done using a model system, and it is to be clarified whether or not S–1 actually has an antioxidative activity in foods containing oils.

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