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Antioxidative catechol-type lignans in the seed coat of tung tree (*Vernicia fordii*) that inhibit the oxidation of drying oil in the endosperm

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Abstract

Tung oil contains approximately 78% α -eleostearic acid (9c, 11t, 13t-octadecatrienoic acid), 9% linoleic acid, and 7% oleic acid. This oil is easily oxidized because of the three conjugated double bonds in α -eleostearic acid; however, it is stable within the tung seed. Previously, we isolated and identified a catechol-type lignan and neolignans from tung seeds, which exhibited potent antioxidant activity. Catechol compounds were specifically present in the seed coat of tung trees. In this study, we quantified the catechol-type lignans in the seed coat of Euphorbiaceae oil-producing plants and found that the plant seeds containing easily oxidized fatty acids contained higher amounts of catechol-type lignans in their seed coat. The antioxidant activity assay of phenolic compounds indicated that catechol-type compounds have higher antioxidant activity than monophenol-type compounds, and that the two hydroxy groups in the *ortho*-position of the benzene ring are important for enhancing antioxidant activity. Analysis of the antioxidant mechanism by ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy showed that isoamericanol A captured the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical and preferentially changed it to the quinone structure. The oil oxidation inhibition test revealed that α -tocopherol inefficiently inhibited the oxidation of tung oil, and that catechol compounds with a higher antioxidative activity than α -tocopherol would be required to prevent the oxidation of tung oil. These results show that tung seeds synthesize catechol-type lignans in the seed coat to prevent the oxidation of tung oil in the endosperm.

Keywords Antioxidant, Catechol, Euphorbiaceae, Lignan, Neolignane, NMR

Introduction

Euphorbiaceae is one of the largest families of flowering plants and consists of over 300 genera and 8,000 species [1]. This family occurs mainly in tropical rainforests and is distributed worldwide under a variety of environmental conditions, from temperate regions to dry deserts [2]. The species are mostly monoecious herbs, shrubs, trees, and sometimes vine- and cactus-like forms that adapt

to their environmental conditions. These plants produce various secondary metabolites, including alkaloids, phorbol esters, cyanogenic glycosides, and toxic proteins. Useful commercial products, such as folk medicine from castor oil plant (*Ricinus communis*) [3], tropical starch from cassava (*Manihot esculenta*), natural rubber from para rubber tree (*Hevea brasiliensis*), drying oil from tung tree (*Vernicia fordii*), candle wax from Chinese tal-low tree (*Triadica sebifera*) [4], and bio-diesel fuel from jatropha (*Jatropha curcas*), are obtained from this family [5, 6]. Euphorbiaceae seed oils generally contain unsaturated and characteristic fatty acids.

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The tung tree is native to southern China and was introduced to Japan in the nineteenth century. Its kernel contains approximately 40% oil, which is not suitable for human consumption because it causes diarrhea. Tung oil has many applications in the paint industry and is used as a raw material for linoleum, resins, oil cloth, and artificial leather [7]. The oil contains approximately 78% α -eleostearic acid (9*c*, 11*t*, 13*t*-octadecatrienoic acid), 9% linoleic acid, and 7% oleic acid [8], and is easily oxidized due to the three conjugated double bonds in α -eleostearic acid. Phorbol esters including 12-*O*-palmityl-13-*O*-acetyl-16-hydroxyphorbol and the monoester 13-*O*-acetyl-16-hydroxyphorbol were isolated from the fruit [9], and the related diterpenes from the leaves exhibited ichthyotoxic effects [10]. A methanolic leaf extract containing tiglic acid ester stimulated insulin secretion in pancreatic β cells [11]. Triterpenoids that were isolated from the leaves exhibited growth inhibitory activity against cancer cells [12]. Lignans, sesquiterpenoids, and cycloneolignans that were isolated from the fruits have been identified as neuroinflammatory inhibitors [13, 14].

Nuclear magnetic resonance (NMR) is a powerful analytical tool for identifying chemical structures and molecular interactions. ^1H and ^{13}C NMR spectroscopy have been used to elucidate the antioxidant molecular mechanisms of phenolic compounds [15]. Sawai et al. reported that when (+)-catechin and (+)-taxifolin were reacted with 2,2-diphenyl-1-picrylhydrazyl (DPPH), the catechol structures in the B-ring changed to *o*-quinone structures [16]. However, the catechol structures in quercetin and ethyl protocatechuate were not oxidized to *o*-quinone.

Previously, we isolated and identified a catechol-type lignan (3,3'-bisdemethylpinoresinol) and catechol-type neolignans (isoamericanol A and americanol A) from the seeds of *Jatropha* [17], which exhibited strong antioxidant activity similar to that of trolox, and found that catechol compounds were specifically present in the seed coat of tung trees [18]. In this study, we quantified the catechol-type lignans in the seed coat of Euphorbiaceae oil-producing plants, evaluated the antioxidant activity of the catechol-type compounds, analyzed their antioxidative mechanism using ^{13}C NMR, and performed an oil oxidation inhibition test to clarify the function of catechol-type lignans.

Materials and methods

Plant materials and chemicals

Tung tree, Chinese tallow (*Triadica sebifera*), and *Jatropha* (*Jatropha curcas*) seeds were collected from a private forest in Sanuki City, Bushozan Park in Takamatsu City, and the farm at Chiang Mai University, respectively. Castor bean (*Ricinus communis*) seeds were purchased from an herbal medicine shop.

Resorcinol, ferulic acid, trolox, α -tocopherol, 3,4-dihydroxybenzoic acid, caffeic acid, and epicatechin were purchased from nakarai tesuque and FUJIFILM Wako Pure Chemical Corporation.

Quantitative HPLC analysis of catechol lignans

The seed coat (ca. 1 g) was separated from the seeds, ground using a mortar and pestle, and extracted with methanol (20 mL $\times$ 3) [18]. The methanolic extract was concentrated to dryness under reduced pressure, purified using a Sep-Pak silica cartridge (2 g, ethyl acetate/hexane = 50% and 100%, 30 mL each fraction), and the 100% fraction was concentrated to dryness. The concentrate was dissolved in 10 mL MeOH, filtered through a 0.45 μm membrane filter, and analyzed on a JASCO LC-2000 plus system equipped with a JASCO UV-2075 plus ultraviolet–visible absorption spectroscopy (UV–Vis) detector (254 nm) using Chromato Pro (ver. 5.0). Separation was performed on a reversed-phase column (ϕ 4.6 $\times$ 250 mm, COSMOSIL, 5C₁₈-MS-II) with a guard column. The mobile phase program started with a linear gradient from 20% MeOH to 80% MeOH over 40 min, then isocratic elution for 50 min. The flow rate was adjusted to 1.0 mL/min, and the column temperature was maintained at 40 $^\circ\text{C}$. The injection volume of the samples and standards was 10 μL . Isoamericanol A, a standard compound, was synthesized by the radical coupling reaction of caffeyl alcohol [18]. A calibration curve was constructed by plotting the peak area against the concentration of the standards (0.2–4 $\mu\text{g}/10 \mu\text{L}$). The amounts of catechol-type lignans were expressed as isoamericanol equivalents.

Antioxidant activities of monophenol and catechol-type compounds

The antioxidant activities of monophenol- and catechol-type compounds were assayed for the scavenging of stable DPPH free radicals according to a previously reported method [17]. The compounds were dissolved in methanol to make a 50 ppm solution. Each of the test sample solutions (0, 30, 60, 90, 120, and 150 μL) was added into a mixture (0.9 mL) of 0.4 mM DPPH solution, 20% methanol aqueous solution, and 0.2 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer solution (1:1:1). The resulting mixtures were shaken on a vortex mixer and allowed to stand for 20 min. The absorbance of the remaining DPPH was determined using an ultraviolet (UV)–visible spectrophotometer at 520 nm. The percentage of DPPH radical inhibition by each sample was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = (1 - A/A_0) \times 100.$$

where A_0 is the absorbance of the mixture without the sample and A is the absorbance of the mixture with the sample after 20 min. The inhibitory concentration that

resulted in 50% scavenging of DPPH radical (IC_{50}) was estimated based on the plot of inhibition versus final concentration of the test samples.

Antioxidant mechanism analysis by ^{13}C NMR

A sample (0.015 mol) was dissolved in acetone- d_6 (0.7 mL) in an NMR tube, and DPPH (0.030 mmol) was added to the NMR tube [16]. The reaction mixture was immediately subjected to ^{13}C NMR (JEOL JMN 500 Hz) and the spectra were compared with those obtained without DPPH.

Oil oxidation inhibition test of the catechol-type lignans

The tung tree seed coat (65 g) was extracted using methanol (120 mL) and evaporated to dryness under reduced pressure. The extract was partitioned between ethyl acetate and water and purified using a Sep-pack silica cartridge (Waters). The 100% ethyl acetate fraction was crystallized in hexane and ethyl acetate solutions (1:1) to obtain the catechol-type lignan fraction. The fraction (58 mg) containing a mixture of isoamericanol A and americanol A was used for the oil oxidation inhibition tests.

Commercial tung oil was purified by column chromatography to remove antioxidant compounds and oxidized fatty acids and bubbled through N_2 gas to remove the residual solvent [19]. α -Tocopherol and the catechol-type lignan fraction (100, 500, and 1,000 ppm) were added to the refined tung oil (10 g) to evaluate the oil oxidation prevent effect. The oils were heated at a constant

temperature of 60 °C in the dark, and the peroxide value (POV), which is used as an indicator of the early stage of oil oxidation, was determined by the JOSC method [20]. The POV (mean $\pm$ standard error (SE)) was expressed as meq/kg oil.

Results and discussion

Figure 1 shows the contents of the three catechol-type lignans in the seed coat of Euphorbiaceae plants. Lignans were the most abundant in the seed coat of the tung tree, followed by castor bean, jatropha, and Chinese tallow. The seed coat of the tung tree contained 511 μ g/g of isoamericanol A, 287 μ g/g of americanol A, and 71.3 μ g/g of 3,3'-bisdemethylpinoresinol. Table 1 shows the fatty acid compositions of the tested plants. Tung tree seeds contain approximately 80% eleostearic acid, which has three conjugated double bonds and tends to be easily oxidized [8]. The main fatty acids in castor beans, jatropha, and Chinese tallow are ricinoleic, oleic, and oleic acids, respectively [21]. The degree of unsaturation of fatty acids in oil plants varies depending on the growing environment and variety; it is generally high in low-temperature growing environments and low in high-temperature growing environments, and plant oil containing conjugated double bonds and hydroxy groups is easily oxidized [22]. These results indicate that Euphorbiaceae plant seeds containing easily oxidized fatty acids have higher amounts of catechol-type lignans in their seed coats.

Table 2 shows the DPPH radical-scavenging activities of phenolic compounds. Gallic acid, a pyrogallol-type

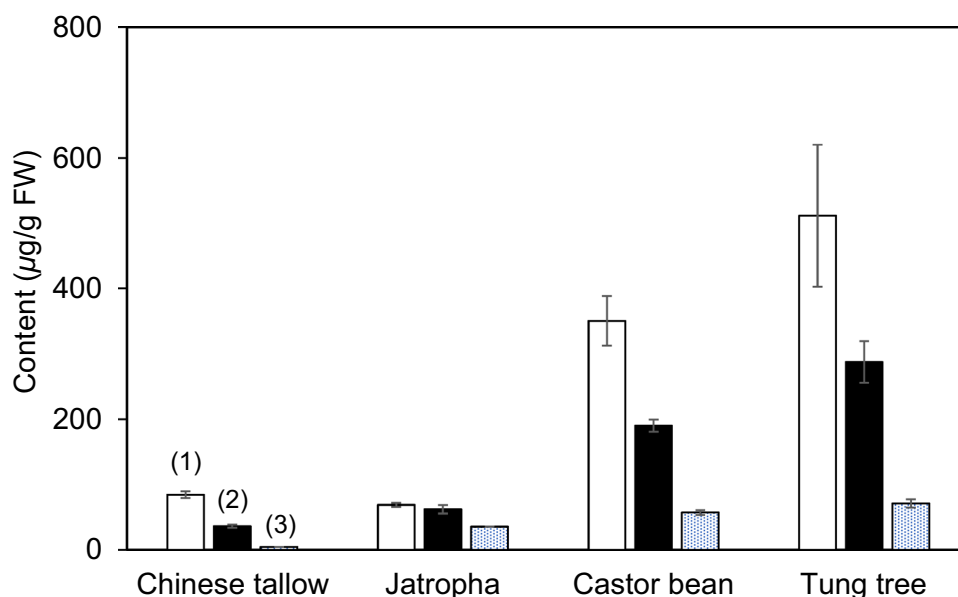


Fig. 1 Contents of three catechol-type lignans in the seed coat of Euphorbiaceae plants. (1) Isoamericanol A, (2) americanol A, and (3) 3,3'-bisdemethylpinoresinol. The data represent the mean of four independent measurements (mean $\pm$ SE)

Table 1 Fatty acid composition (%) of oils produced by Euphorbiaceae plants

		Chinese tallow	Jatropha	Castor bean	Tung tree
Palmitic acid	(16:0)	26.78	14.2		3.32
Palmitoleic acid	(16:1)		1.4		
Stearic acid	(18:0)	3.87	6.9		2.47
Oleic acid	(18:1)	31.61	43.1	4.29	6.82
Ricinoleic acid	(18:1)			88.2	
Linoleic acid	(18:2)	22.25	34.4	7.47	9.04
Linolenic acid	(18:3)	9.98			
Eleostearic acid	(18:3)	conjugate double bonds			78.4

Table 2 The DPPH radical scavenging activity of phenols

		IC ₅₀ (mol/L)
Resorcinol-type	Resorcinol	484.4 ± 16.00
Monophenol-type	Ferulic acid	32.3 ± 0.40
	Trolox	23.7 ± 0.42
	α-Tocopherol	23.0 ± 1.40
Catechol-type	3,4-Dihydroxybenzoic acid	22.8 ± 1.86
	Methyl caffeate	15.6 ± 0.60
	Caffeic acid	12.9 ± 0.42
	Epicatechin	11.7 ± 0.51
	Isoamericanol A	9.57 ± 0.30
Pyrogallol-type	Gallic acid	7.66 ± 0.38

compound, exhibited the highest antioxidant activity among all the tested compounds [23]. Isoamericanol A exhibited the second highest antioxidant activity, followed by epicatechin and caffeic acid. Catechol-type compounds showed higher antioxidant activities than monophenol-type compounds. However, resorcinol, which has two hydroxy groups that are not in the adjacent position on the benzene ring, exhibited the lowest antioxidant activity. These results indicate that the two hydroxy groups in the *ortho*-position of the benzene ring are important for enhancing the antioxidant activity [24]. Resorcinol was reacted with DPPH in acetone-d₆ and immediately subjected to ¹³C NMR spectroscopy (Fig. 2).

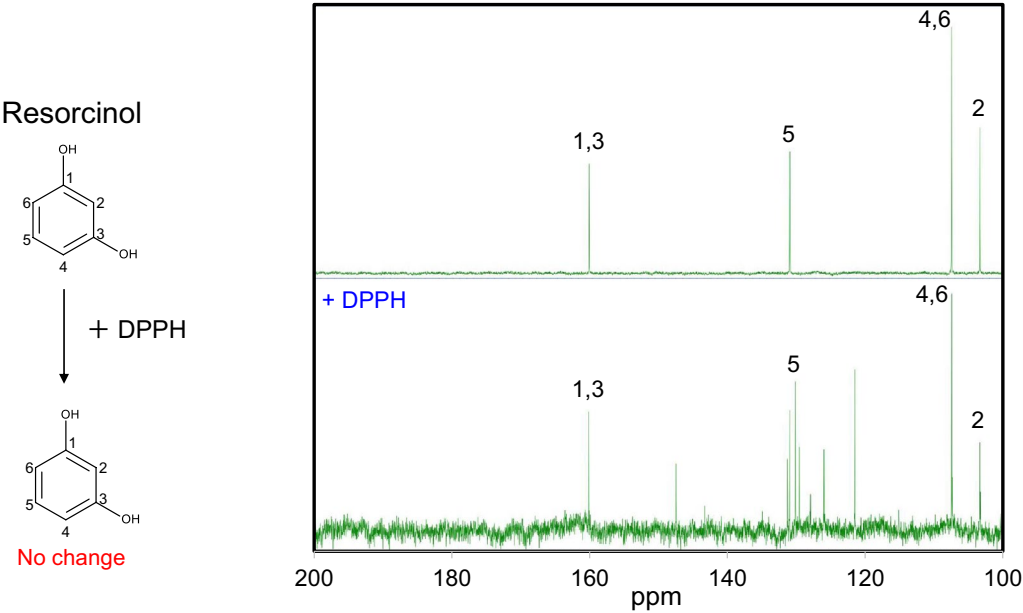


Fig. 2 Spectra of resorcinol with and without DPPH

The spectra obtained with DPPH were compared with those obtained without DPPH. No significant changes were observed in the signals of resorcinol with DPPH, indicating that resorcinol barely captured DPPH radical, supporting the results in Table 2. In the case of ferulic acid with DPPH (Fig. 3), a conjugated double bond signal (C7) and a carboxylic acid signal slightly decreased relative to those in the spectra without DPPH, and a small new signal (δ 176.0) appeared in the spectra with DPPH, indicating that ferulic acid captured a little DPPH radical and ferulic acid adopted some resonance structures.

A significant change was observed in the signals derived from the catechol ring of isoamericanol A in the presence of DPPH (Fig. 4). The signals derived from C-3 and C-4 disappeared, and the appearance of two distinct carbonyl signals (δ 181.3 and 180.4) indicated that the catechol ring changed to the quinone structure [16]. Few changes were observed in the other signals derived from the benzodioxane ring. The spectroscopic analysis revealed that isoamericanol A captured DPPH radical and preferentially changed to the quinone structure. Similar changes were observed for the reaction of epicatechin with DPPH [25, 26]. The signals derived from C-3' and C-4' almost disappeared, and the other B-ring signals (C-1', 2', 5', and 6') appear to shift to a lower magnetic field (Fig. 5). However, it is difficult to completely assign the signals. The appearance of two clear new carbonyl signals (δ 181.3 and 180.3) indicated that the B-ring changed

to the quinone structure. Meanwhile, little change was observed in the other signals derived from the A- and C-rings.

However, not all catechol compounds can adopt quinone structures [16]. In caffeic acid observation (Fig. 6), the C-3 and C-4 signals (δ 148.6 and 146.2 ppm) in the catechol ring and a carboxylic acid signal (δ 168.3 ppm) almost disappeared, other signals of the catechol ring (C-1, 2, 5, and 6) appear to shift to a lower magnetic field, and the appearance of two new signals (δ 181.0 and 180.6) in the spectra with DPPH, but these signals are weak, indicating that caffeic acid captured the DPPH radical and adopted resonance structures. Similar results were obtained for methyl caffeate and DPPH. Sawai et al. reported that (+)-catechin and (+)-taxifolin tend to change to the *o*-quinone structure in the presence of the DPPH radical; however, ethyl protococatechuate and quercetin can easily form resonance structures, even though these compounds have a catechol group [16]. They suggested that the tendency of the compound to form the *o*-quinone structure was related to the presence or absence of a conjugated double bond or a carbonyl bond next to the catechol group. Isoamericanol A has a benzoquinone structure, but any conjugated double bond or carbonyl bond next to the catechol group would lead to the characteristic structure preferentially changing to form the *o*-quinone structure by radical oxidation (Fig. 7).

Figure 8 shows the effect of α -tocopherol and the catechol-type lignan fraction on the oxidation of refined tung

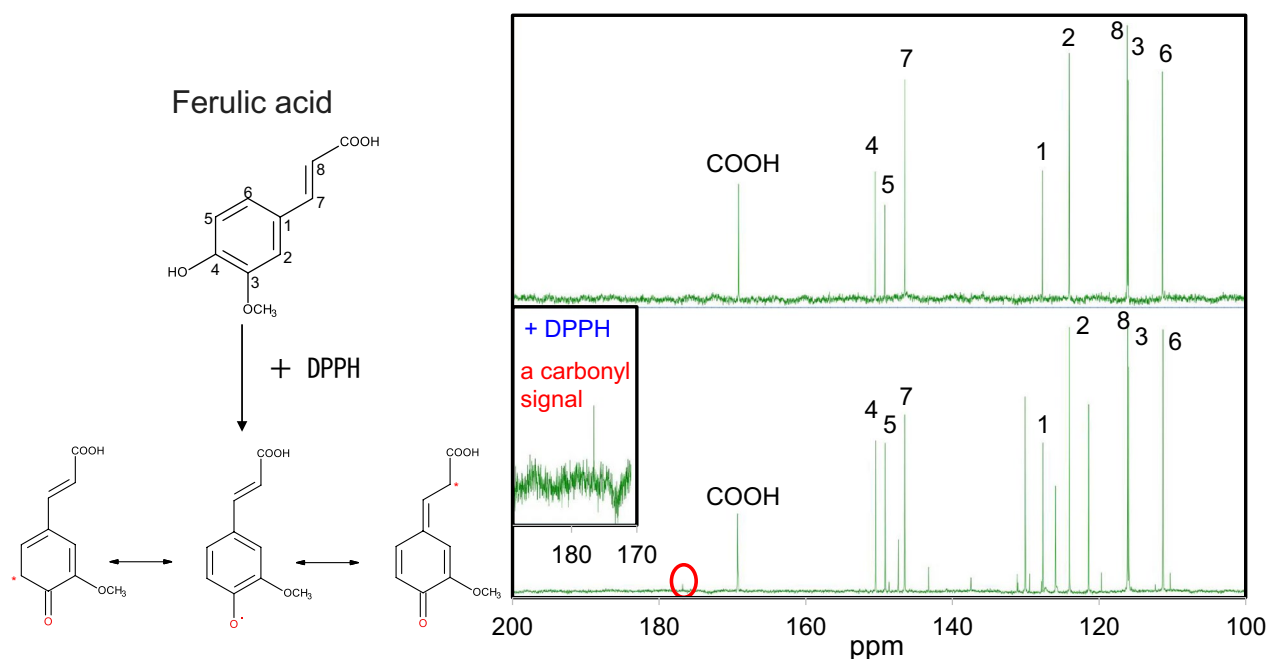


Fig. 3 Spectra of ferulic acid with and without DPPH

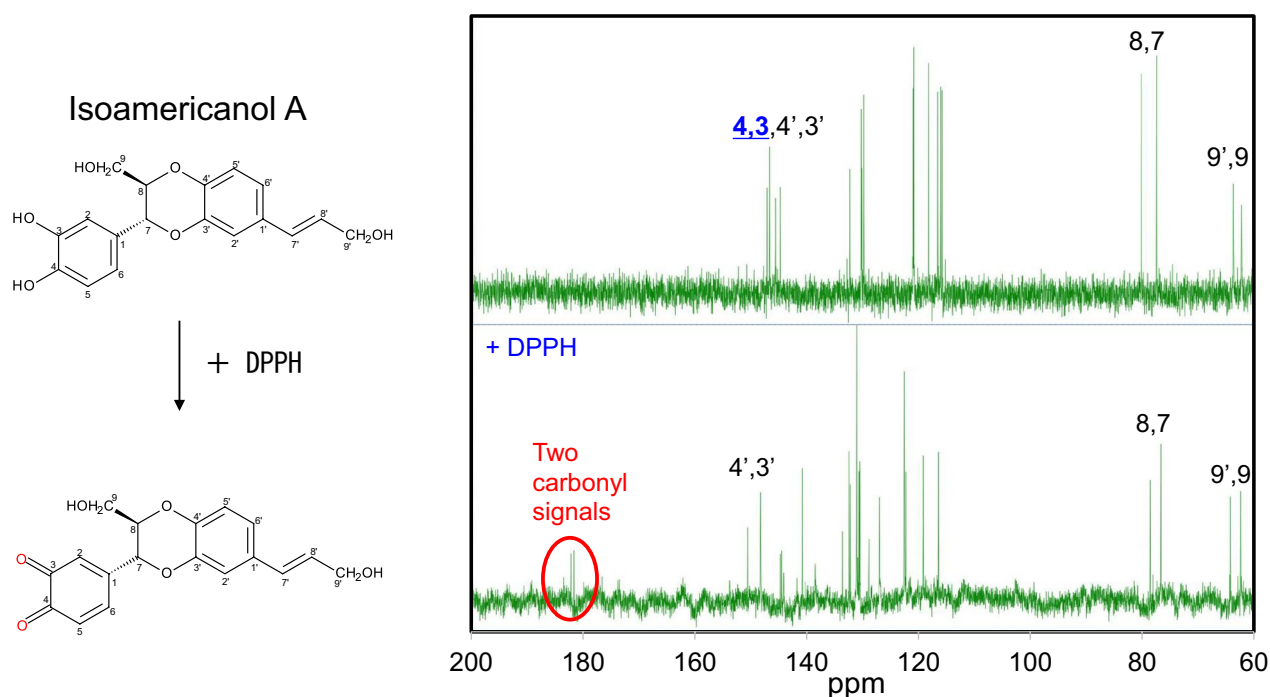


Fig. 4 Spectra of isoamericanol A with and without DPPH. Missing signals are underlined

oil. In the case of the tung oil with α -tocopherol, the POV values increased with increasing tocopherol concentration (100–1000 ppm). Some researchers have reported that α -tocopherol rather exhibited pro-oxidant effect on easily oxidized oils. Flaxseed oil, for example, is a drying oil and a rich source of essential fatty acids, such as linolenic acid (C18:3, 50–60%) [27]. α -Tocopherol acted as a pro-oxidant and was unable to protect the flaxseed oil from oxidation. Docosahexaenoic acid (DHA, C22:6) is a long-chain omega-3 fatty acid found in fish oil [28]. Green tea extract enhanced the oxidation stability of DHA-rich oil, whereas α -tocopherol had no significant effect on the PV during storage at 30 °C, but resulted in a significantly higher anisidine value. Whether α -tocopherol acts as an antioxidant or a pro-oxidant appears to depend not only on the fatty acid composition of oil, but also on α -tocopherol concentration [29], temperature, UV, and etc.

However, in the case of tung oil with a catechol-type lignan fraction, the POV values were inhibited at higher concentrations. These results indicate that α -tocopherol inefficiently inhibits the oxidation of tung oil, and that catechol compounds with higher antioxidative activities than α -tocopherol are required to prevent the oxidation of tung oil. Therefore, the tung tree synthesizes catechol-type lignans in the seed coat to prevent oxidation of tung oil in the endosperm.

Antioxidants are used to improve food quality by decreasing the rate of lipid oxidation in oil-containing

foods [30]. Tocopherols are natural antioxidant compounds found mainly in vegetable oils [31]. They are classified into four derivatives (α , β , γ , and δ) depending on the position and number of methyl groups on the chromanol ring. α -Tocopherol is widely known as vitamin E, which is a more stable and more effective antioxidant than the other tocopherols. α -Tocopherol that has been synthesized or recovered from residues of soybean, rapeseed, and palm oil has been used as food additives to stabilize edible fats and oils [29, 32]. Common edible oils such as olive oil, rapeseed oil, and palm oil are composed of oleic, linoleic, and linolenic acids. These fatty acids have either one double bond or non-conjugated double bonds. α -Tocopherol would be sufficient to suppress the oxidation of the edible oils that are composed of these fatty acids. However, tung oil is composed of α -eleostearic acid, which has three conjugated double bonds; therefore, tung oil oxidizes easily and is classified as a drying oil. Drying oils, such as tung oil, linseed oil, castor oil, and walnut oil, are widely used for oil paints, varnishes, and inks. These oils are easily oxidatively polymerized, harden into a tough form, and form a solid film at room temperature [33, 34]. Oils with unsaturated fatty acids undergo oxidative polymerization in air, resulting in a hundreds to thousands of times increase in molecular weight.

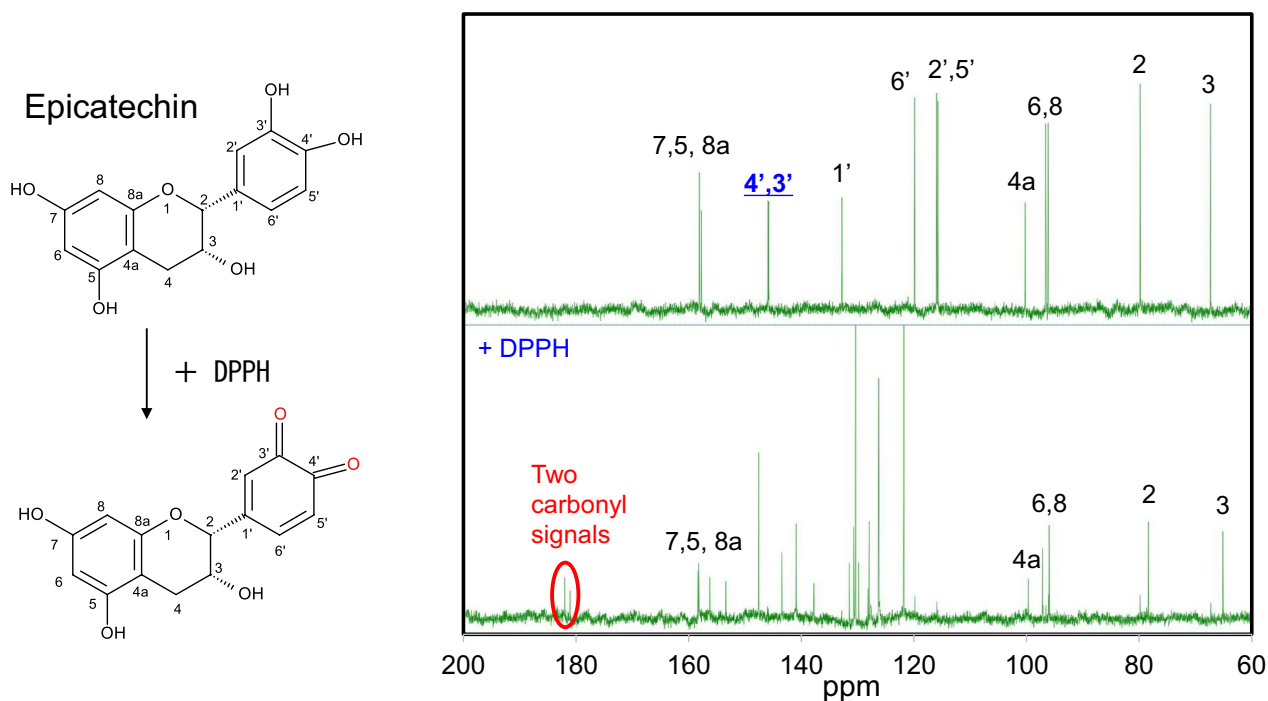


Fig. 5 Spectra of epicatechin with and without DPPH. Missing signals are underlined

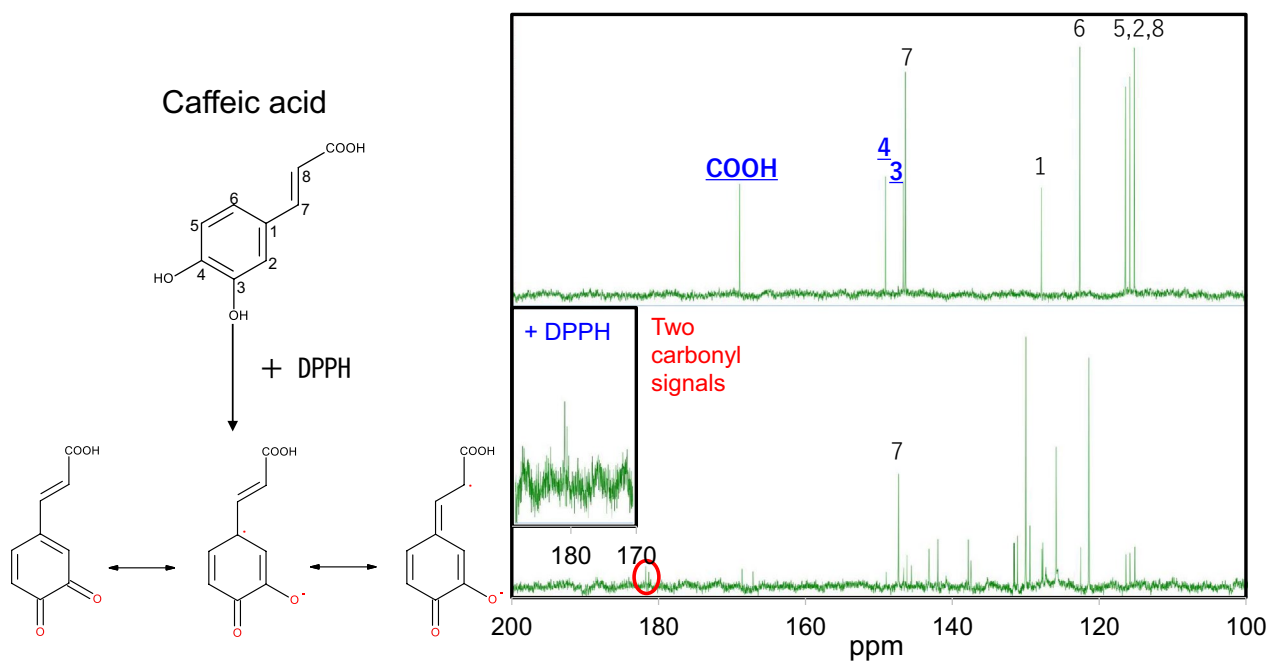


Fig. 6 Spectra of caffeic acid with and without DPPH. Missing signals are underlined

However, little attention has been paid to the mechanisms by which these drying oils remain stable inside plant seeds.

Conclusions

Reactive oxygen radicals generated in living organisms randomly oxidize lipids, proteins, and nucleic acids, resulting in the induction of cancer, arteriosclerosis, and other cardiac diseases. The generation of reactive oxygen

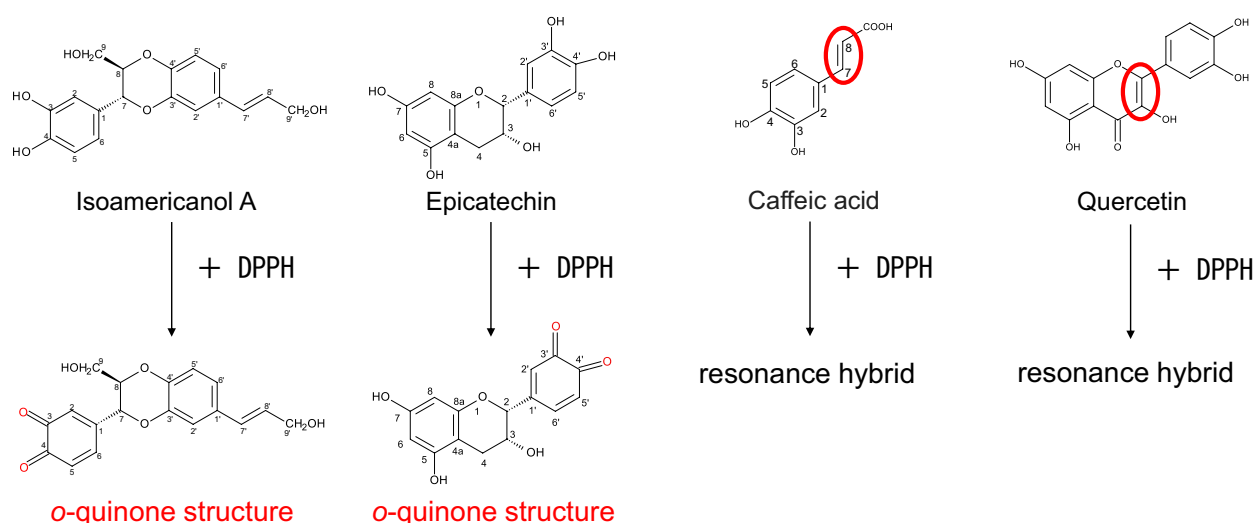


Fig. 7 Tendency of an *o*-quinone structure by catechol-type compounds depended on the presence or absence of a conjugated double bond next to the catechol group. The result of quercetin was based on the reference [16]

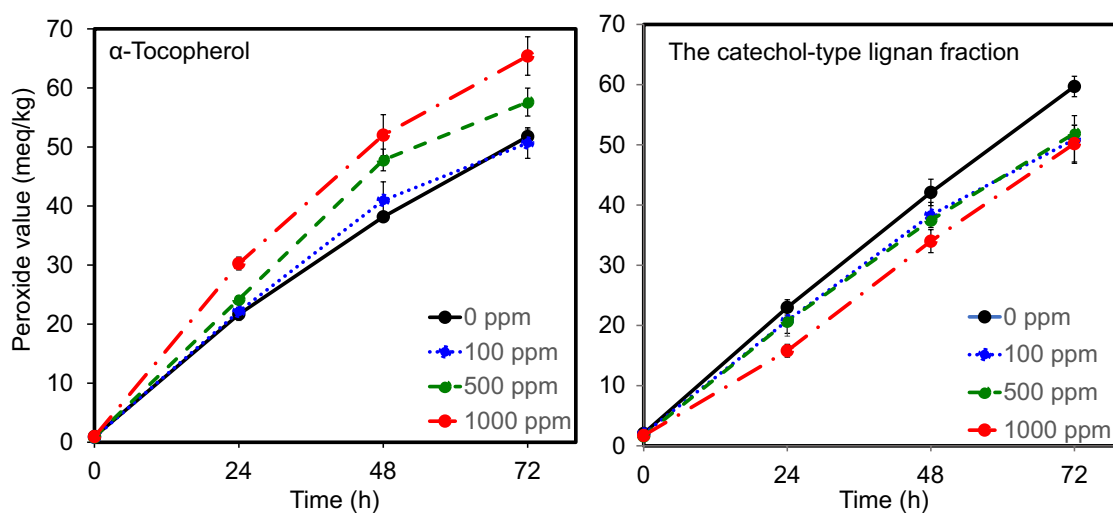


Fig. 8 Peroxide values (mean $\pm$ SE) of refined tung oil with different concentrations of α -tocopherol and the catechol-type lignan fraction after storage at 60 $^{\circ}$ C ($n=3$)

radicals and antioxidative mechanisms that scavenge these radicals have been studied in many fields, including chemistry, biochemistry, and medicine. In the food industry, oxidation of fats and oils is a major factor in the deterioration of food, and preventing lipid oxidation is an important issue. In this study, we showed that tung trees synthesize catechol-type lignans in their seed coats to prevent the oxidation of tung oil, which is the most easily oxidized oil among terrestrial plants. This study

expands our knowledge of antioxidant mechanisms in living organisms.

Abbreviations

DHA	Docosahexaenoic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
MES	2-(<i>N</i> -Morpholino) ethanesulfonic acid
NMR	Nuclear magnetic resonance
POV	Peroxide value
SE	Standard error
UV-Vis	Ultraviolet-visible absorption spectroscopy

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Author contributions

TS designed this study and wrote the manuscript. KN measured the antioxidative activities and ^{13}C NMR. TK discussed the results and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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