Antioxidative and Cytotoxic Compounds Extracted from the Sap of *Rhus succedanea*

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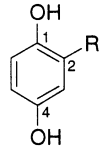
Two new antioxidative and cytotoxic compounds, 10′(*Z*),13′(*E*),15′(*E*)-heptadecatrienylhydroquinone (**1**) and 10′(*Z*),13′(*E*)-heptadecadienylhydroquinone (**2**), as well as the known 10′(*Z*)-heptadecenylhydroquinone (**3**), were isolated from an EtOH extract of the sap of *Rhus succedanea*. The structures were elucidated by spectral analyses. These compounds showed antioxidative and cytotoxic activities against five cancer cell lines.

The lacquer trees, *Rhus succedanea* L. (Anacardiaceae), grown in Puli, Nantao, Taiwan, originated from Vietnam and are different from *Rhus vernicifera* mainly grown in Mainland China, Korea, and Japan.[1−3] The sap collected from lacquer trees, called oriental or natural lacquer, has been used for adhesives and coatings for more than 5000 years in China and Japan. Since the Min Dynasty of China, as recorded in "Bern Chao Gan Mo", the dried lacquer after grinding into powder has been used as a folk medicine for various therapeutic purposes.[4]

Antioxidant-directed fractionation of the 80% ethanol extract from the sap of *Rhus succedanea* by HPLC afforded two new compounds, **1** and **2**, as well as **3**.[5] Herein, we describe the isolation, structural elucidation, and antioxidative and cytotoxic activities of these compounds.



1: R = 10′(Z),13′(E),15′(E)-heptadecatrienyl
2: R = 10′(Z),13′(E)-heptadecadienyl
3: R = 10′(Z)-heptadecenyl

10′(*Z*),13′(*E*),15′(*E*)-Heptadecatrienylhydroquinone (**1**) showed a molecular ion at *m*/*z* 342.2588 by HREIMS, which was consistent with the molecular formula of $C_{23}H_{34}O_2$. A base peak in the mass spectrum was observed at *m*/*z* 123, which corresponded to the dihydroxytropylium ion $C_7H_5(OH)_2^+$. The ^{13}C NMR spectrum revealed that the substituents were two phenolic hydroxyl groups (δ 144.3 and 145.9) and a carbon chain (δ 130.7). According to the molecular weight, a carbon chain containing 17 carbons with three double bonds was suggested. Three aromatic protons at δ 6.56 (1H, d, J = 2.8 Hz), 6.58 (1H, d, J = 7.2 Hz), and 6.61 (1H, dd, J = 7.2, 2.8 Hz) comprised a 1,2,4-trisubstituted benzene structure. The distinct NOE cross-peak between aromatic H-3 (δ 6.56) and benzylic H-1′ (δ 2.58) together with the $^1H-^{13}C$ long-range correlation between benzylic H-1′ and aromatic C-1 (δ 144.3) and C-3 (δ 121.9) (Figure 1) showed a heptadecatrienylhydroquinone skeleton. The position of the double bonds in the side chain was determined by COSY, HMQC, and HMBC experiments. A doublet methyl at δ 1.70 (J = 6.4 Hz, H-17′) coupled with an olefinic proton at δ 5.55 (m, H-16′) indicated a double bond between C-15′ and C-16′. The other two double bonds were suggested to be at carbons 10′ and 11′ as well as 13′ and 14′ due to a quartet methylene at δ 2.05 (J = 6.8 Hz, H-9′) and a triplet methylene at δ 2.79 (J = 6.8 Hz, H-12′) together with the $^1H-^{13}C$ long-range correlations between H-12′ (δ 2.79) and C-10′ (δ 131.8), C-11′ (δ 128.1), C-13′ (δ 130.7), and C-14′ (δ 131.8). The geometry of these double bonds was determined as follows. Based on Rossi's method,[6] the upfield-shifted carbon signal of allylic C-9′ (δ 27.1 in $CDCl_3$) and the downfield-shifted carbon signals of C-12′ (δ 30.3 in $CDCl_3$) and C-17′ (δ 18.0 in $CDCl_3$) in **1** with respect to the analogous carbons C-9′ and C-12′ (δ 29−30) and C-17′ (δ 14) in saturated heptadecylcatechol revealed the double-bond configurations as 10′(*Z*), 13′(*E*), and 15′(*E*). Furthermore, an apparent doublet with J = 9.6 Hz for H-10′ by irradiation of H-9′, a doublet with J = 13.8 Hz for H-13′ by irradiation of H-12′, and a doublet with J = 12.4 Hz for H-16′ by irradiation of H-17′ supported the stereochemistry of the double bonds between carbons 10′ and 11′; 13′ and 14′; and 15′ and 16′ as *Z*, *E*, and *E*, respectively.

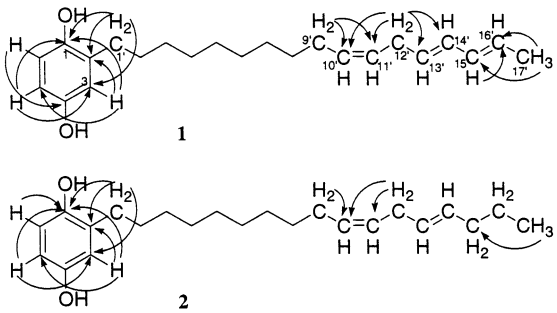


Figure 1. The major HMBC correlations of compounds **1** and **2**.

10′(*Z*),13′(*E*)-Heptadecadienylhydroquinone (**2**) exhibited the molecular formula $C_{23}H_{36}O_2$, two mass unit more than **1**. The similarity between the ^1H and ^{13}C NMR spectra of compounds **1** and **2** revealed that **2** possessed two double bonds in the C_{17} side chain. The chemical shift and splitting pattern of H-9′ (δ 2.04, q, J = 6.8 Hz), H-12′ (δ 2.73, t, J = 6.0 Hz), and H-15′ (δ 1.96, q, J = 6.0 Hz) as well as the ^3J HMBC correlation between H-17′ (δ 0.89) and C-15′ (δ 35.8) indicated two double bonds located between C-10′ and C-11′; and C-13′ and C-14′ (Figure 1). The presence of an NOE between H-9′ and H-12′ revealed the *cis* configuration of the C-10′−C-11′ double bond, whereas the absence of an NOE between H-12′ and H-15′ disclosed the *trans* configuration of the C-13′−C-14′ double bond.

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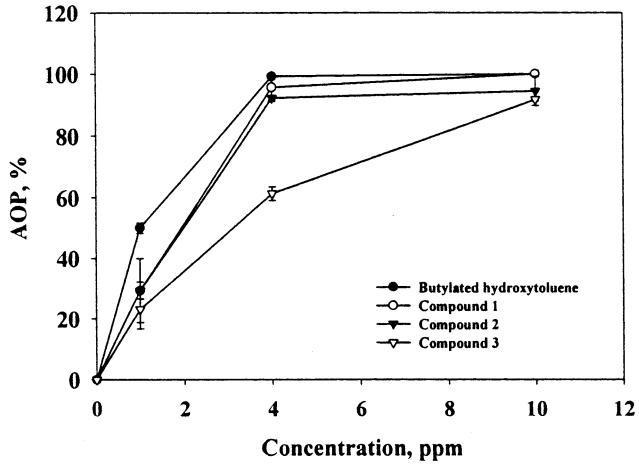

Figure 2. Antioxidative potency (AOP) of the hydroquinone compounds **1**, **2**, and **3** extracted and purified from the sap of *Rhus succedanea*.

Table 1. Cytotoxicity of the Hydroquinone Compounds **1**−**3** from the Sap of *Rhus saccedanea* toward Five Cancer Lines[a]

	cell line and IC_{50} in μg/mL				
compound	HeLa	Huh7	HCT116	LoVo	C6
1	2.8	3.9	2.0	4.5	0.9
2	4.6	6.0	3.5	5.6	1.0
3	4.7	6.4	3.4	2.9	1.1

[a] HeLa = human cervix epithelioid carcinoma; Huh7 = human hepatoma cell line; HCT116 = human colorectal cancer cell line; LoVo = human colon adenocarcinoma; C6 = rat C6 glioma cells.

The three hydroquinones **1**−**3** and a known antioxidant, butylated hydroxytoluene (BHT), were evaluated using an iron/ascorbate system with linoleic acid as substrate for antioxidative potency (AOP) determination. The results are shown in Figure 2. In comparison, except compound **3** at the concentration of 4 ppm, the AOPs of the hydroquinones **1**−**3** were close to that of BHT.

Hydroquinones **1**−**3** were also evaluated using an in vitro anticancer assay. These compounds exhibited significant cytotoxic activity against five cancer cell lines including cervix epithelioid carcinoma (HeLa), hepatoma cell line (Huh7), colorectal cancer cell line (HCT116), colon adenocarcinoma (LoVo), and rat C6 glioma cells. The IC_{50} values against the cancer cell lines were 2.0−4.5 mg/mL for compound **1**, 3.5−6.0 μg/mL for compound **2**, and 2.9−6.4 μg/mL for compound **3**, respectively, depending on the cancer cell type (Table 1).

Experimental Section

General Experimental Procedures. The UV spectra were recorded in $CHCl_3$. The IR spectra were measured as thin film on KBr. ^{1}H and ^{13}C NMR spectra were measured in CD_3-OD. Low- and high-resolution MS were performed in the EI mode. High-performance liquid chromatographic (HPLC) analyses were monitored at 254 nm, and a reversed-phase C_{18} column or semipreparative column was used.

Plant Material. The sap, natural lacquer, was obtained from Longnan Natural Lacquer Museum, Puli, Nantao, Taiwan. The lacquer was harvested from the Lac trees, *Rhus succedanea*, grown in the Puli area by cutting the trunk skin of lacquer trees into stripes and collecting the outflow. A voucher specimen was deposited in the Herbarium of National Chiayi University, Taiwan.

Extraction and Isolation. An aliquot of the lacquer (10 g) was dissolved and mixed thoroughly with 90 mL of 80% EtOH at ambient temperature. The upper layer was withdrawn and centrifuged (8000*g*, 5 min). The supernatant was diluted 20-fold with 80% MeOH and subjected to HPLC analysis run with a solvent gradient of 50−90% aqueous MeOH (v/v). The flow rate and injection volume were 1 mL/min and 20 μL, respectively. Three peaks with potent antioxidative activity were observed. The three active fractions were separated by semipreparative HPLC using MeOH/H_2O (80:20, v/v) with 3 mL/min flow rate and 2.5 mL injection volume. These subfractions were purified by running through the same column (MeOH/H_2O, 85:15, v/v). Compounds were lyophilized using a freeze-drier. The yields for compounds **1**, **2**, and **3** were 23.0, 31.5, and 31.5 mg/g of sap, respectively.

10′(*Z*),13′(*E*),15′(*E*)-Heptadecatrienylhydroquinone (1): pale yellow oil; UV λ_{max} (log ϵ) 247 (4.02), 277 (3.64), 319 (2.93) nm; IR ν_{max} 3499, 3012, 2925, 2852, 1594, 1475, 1278, 986, 732 cm^{-1}; ^{1}H NMR δ 1.30 (12H, m, H-3′-8′), 1.58 (2H, quintet, J = 7.6 Hz, H-2′), 1.70 (3H, d, J = 6.4 Hz, H-17′), 2.05 (2H, q, J = 6.8 Hz, H-9′), 2.58 (2H, t, J = 7.6 Hz, H-1′), 2.79 (2H, t, J = 6.8 Hz, H-12′), 5.37 (1H, m, H-11′), 5.41 (1H, m, H-10′), 5.49 (1H, m, H-13′), 5.55 (1H, m, H-16′), 5.99 (2H, m, H-14′ and -15′), 6.56 (1H, d, J = 2.8 Hz, H-3), 6.58 (1H, d, J = 7.2 Hz, H-6), 6.61 (1H, dd, J = 7.2, 2.8 Hz, H-5); ^{13}C NMR δ 18.1 (C-17′), 28.0 (C-9′), 30.6−30.8 (C-3′−8′), 31.1 (C-1′ and -2′), 31.2 (C-12′), 113.6 (C-5), 120.1 (C-6), 121.9 (C-3), 127.7 (C-16′), 128.1 (C-11′), 130.7 (C-2 and -13′), 131.8 (C-10′ and -14′), 132.9 (C-15′), 144.3 (C-1), 145.9 (C-4); EIMS m/z (rel int) 342 M$^+$ (12), 123 (100); HREIMS m/z 342.2588 (calcd for $C_{23}H_{34}O_2$, 342.2559).

10′(*Z*),13′(*E*)-Heptadecadienylhydroquinone (2): colorless oil; UV λ_{max} (log ϵ) 242 (3.40), 274 (3.41) nm; IR ν_{max} 3498, 3013, 2925, 2853, 1475, 1279, 965, 731 cm^{-1}; ^{1}H NMR δ 0.89 (3H, t, J = 7.6 Hz, H-17′), 1.31 (14H, m, H-3′-8′ and H-16′), 1.58 (2H, quintet, J = 7.2 Hz, H-2′), 1.96 (2H, q, J = 6.0 Hz, H-15′), 2.04 (2H, q, J = 6.8 Hz, H-9′), 2.57 (2H, t, J = 7.2 Hz, H-1′), 2.73 (2H, t, J = 6.0 Hz, H-12′), 5.38 (4H, m, H-10′, -11′, -13′, and -14′), 6.55 (1H, d, J = 2.4 Hz, H-3), 6.58 (1H, d, J = 7.2 Hz, H-6), 6.61 (1H, dd, J = 7.2, 2.4 Hz, H-5); ^{13}C NMR δ 14.0 (C-17′), 28.0 (C-9′), 30.3−30.7 (C-3′−8′ and C-16′), 31.0 (C-1′), 31.1 (C-2′), 31.3 (C-12′), 35.8 (C-15′), 113.6 (C-5), 120.1 (C-6), 121.9 (C-3), 128.8 (C-11′), 129.8 (C-13′), 130.7 (C-2), 131.3 (C-10′), 131.4 (C-14′), 144.3 (C-1), 145.9 (C-4); EIMS m/z (rel int) 344 M$^+$ (6), 123 (100); HREIMS m/z 344.2714 (calcd for $C_{23}H_{36}O_2$, 342.2715).

Determination of Antioxidative Activity. The procedure using an iron/ascorbate system was reported by Hsu.[7] In a beaker (50 mL), 100 mg of linoleic acid, 1.0 g of Tween 20, and 20 mL of Tris-buffer (pH 7.4, 50 mM) were combined, gently shaken, and emulsified with a sonicator for 2 min. An iron/ascorbate solution containing 30 μM $FeCl_3$ and 200 μM ascorbic acid in the Tris-buffer (pH 7.4, 50 mM) was prepared daily. In a series of 1.5 mL microfuge tubes was added 0.5 mL of the emulsified linoleic acid, 0.5 mL of the iron/ascorbate solution, and 0.1 mL of MeOH containing various concentrations of the three compounds or BHT including 0, 10, 40, and 100 ppm (μg/mL) (resulting in ca. 0, 1, 4, and 10 ppm of concentration after combination). Prior to incubation, the tubes were immersed and kept in an ice bath. To initiate reaction, the mixtures were mixed by hand (gently to prevent foaming) and placed into the wells of a thermal block set at 37 °C for 5 and 30 min. After incubation, the conjugated diene hydroperoxide (CDHP) content was determined spectrophotometrically. A 0.1 mL sample of the resulting solution was withdrawn and mixed with 2.4 mL of MeOH, and the absorbance (A) was measured at 234 nm. Aliquots (0.1 mL) of MeOH without antioxidant and MeOH containing 2000 ppm of BHT were introduced and incubated concurrently as blank and control. All test data were the average of triplicate analyses. The percentage of AOP (capacity to inhibit peroxide formation in linoleic acid) was calculated as follows.

$$\text{AOP (\%)} = (1 - A_{234\text{nm}} \text{ with antioxidant}/A_{234\text{nm}} \text{ without antioxidant}) \times 100$$

Determination of Cytotoxicity. Four human cancer cell lines, HeLa, Huh7, HCT116, and LoVo, and rat C6 glioma cells were used to assess the cytotoxicity of **1**−**3**. Cell lines, except

Huh7 cells,[8] were purchased from the American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, and 100 mg/mL streptomycin at 37 °C under 5% CO_2. Cells were grown in 96-well plates (2000 cells/well) with or without the addition of compound **1**, **2**, or **3** for 72 h. An acid phosphatase assay was then used to quantitate the viable cells.[8,9]

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