Isolation of Antioxidative Compounds from Brazilian Propolis: 3,4-Dihydroxy-5-prenylcinnamic Acid, a Novel Potent Antioxidant

Katsuhiro HAYASHI, Sadaaki KOMURA, Noriko ISAJI, Nobuko OHISHI, and Kunio YAGI*

Institute of Applied Biochemistry, Yagi Memorial Park, Mitake, Gifu 505–0116, Japan.
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One new and 11 previously known antioxidative compounds were isolated from Brazilian propolis. The new compound was determined as 3,4-dihydroxy-5-prenylcinnamic acid (3-[3,4-dihydroxy-5-(3-methyl-2-butenyl)-phenyl]-2-(*E*)-propenoic acid) by various physical analyses (MS, IR, ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR). The inhibitory activity of each compound against peroxidation of linoleic acid in a micelle solution was measured. We found that the novel compound possessed the highest potency (IC_{50}, 0.17 μM) among them and was more effective than butylated hydroxytoluene (BHT; IC_{50}, 0.36 μM) under the experimental conditions employed. Among the isolated antioxidative compounds, 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C; IC_{50}, 0.44 μM) was found to be most abundant in Brazilian propolis.

Key words 3,4-dihydroxy-5-prenylcinnamic acid; propolis; antioxidant; 3,5-diprenyl-4-hydroxycinnamic acid; lipid peroxidation; linoleic acid

Propolis is a resinous material that honeybees produce from exudates of various plants and beeswax in a beehive, and has been used for folk medicines and foods since ancient times in many parts of the world.[1)] Propolis has been known to possess antibacterial,[2)] antiviral,[3)] antitumoral,[4)] antioxidative,[5,6)] immunomodulatory,[7)] and other beneficial properties.[1,6)] These biological and pharmaceutical effects and chemical constituents of propolis have been mainly studied on samples of Central and Eastern European propolis.[1—8)] On the other hand, Brazilian propolis recently has been widely marketed in various foods and beverages with the intention of preserving or improving human health. There are many preparations with regional differences that may be attributed to different flora. Investigations on chemical and biological properties of Brazilian propolis started within the last 5—6 years,[9—15)] in comparison with the earlier ones on European propolis.

In the present report, we describe the isolation and characterization of antioxidants in Brazilian propolis in terms of a lipid peroxidation assay, according to the method of Kharasch[16)] except that 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) is used as oxidant.[17)] Especially we report the isolation, elucidation of structure, and antioxidative activity of a new compound, 3,4-dihydroxy-5-prenylcinnamic acid (3-[3,4-dihydroxy-5-(3-methyl-2-butenyl)phenyl]-2-(*E*)-propenoic acid).

Results and Discussion

Isolation of Antioxidative Compounds from Propolis We preliminarily examined various ethanol–water mixtures for their extraction efficiency and inhibition of peroxidation of linoleic acid. Among them, use of 70% ethanol as a solvent for extraction afforded a high extract yield (44.5%, expressed as a percent of the amount of dried extract to that of the applied crude propolis) and high antioxidative activity (IC_{50}, 0.4 μg/ml). Moreover, resinous compounds in the extract derived from propolis, that were not desirable for our study, were decreased under this condition. On the other hand, the water extract gave a lower yield (11.1%), even though the inhibitory activity (IC_{50}, 0.4 μg/ml) was similar to that of 70% ethanol extract. Therefore, 70% ethanol was selected as a solvent in the present experiment.

Propolis was cut into small pieces and extracted with 70% ethanol for one day under stirring at room temperature. After filtration and concentration *in vacuo* of the extract, the residue was partitioned with ether and water to give an ether extract. The ether extract was chromatographed on a silica gel column to give 3 fractions (Fr.1—Fr.3), and then these fractions were repeatedly separated by a combination of different kinds of chromatographies, assessing antioxidative activity by monitoring the inhibitory activity against peroxidation of linoleic acid to give the 12 compounds. Fraction 1 afforded 4′,6-dimethoxy-3,5,7-trihydroxyflavone (betuletol, **1**),[18)] kaempferide (**2**), 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C, **3**),[10,19,20)] 4-dihydrocinnamoyloxy-3-prenylcinnamic acid (**4**),[10)] and 6-(2-carboxyethenyl)-2,2-dimethyl-2*H*-1-benzopyran (**5**).[10,21)] 4-Hydroxy-3-prenylcinnamic acid (drupanin, **6**),[19,22)] 4-hydroxy-3-prenylbenzoic acid (**7**),[23)] and 6-methoxykaempferol (**8**)[18,24)] were obtained from fraction 2. Finally, separation of fraction 3 gave 4-hydroxy-3-(*E*)-(4-hydroxy-3-methyl-2-butenyl)-5-prenylcinnamic acid (capillartemisin A, **9**),[25)] 3-methylkaempferol (**10**),[26)] kaempferol (**11**), and a new compound, 3,4-dihydroxy-5-prenylcinnamic acid (**12**) (Fig. 1). Two known compounds **2** and **11** were identified by direct comparison of their spectroscopic properties with each authentic sample. Other known compounds (**1**, **3**—**10**) were identified by comparison of their physical data (mp, MS, HPTLC, IR, UV, ^{1}H- and ^{13}C-NMR) with data on them in the literature. In order to clarify the chemical structures of all of the above isolated compounds, we employed 2D-NMR spectra [^{13}C–^{1}H correlation spectroscopy (^{13}C–^{1}H COSY), heteronuclear multiple bond correlation spectroscopy (HMBC), and nuclear Overhauser effect spectroscopy (NOESY)].

Structure of Compound 12 Physical data of compound **12** obtained as a pale yellow powder are as follows: mp 178—180 °C; FAB-MS (positive mode) *m/z*: 249 $(M+H)^+$; HPTLC Silica gel 60 F_{254} *Rf*: 0.19, chloroform–methanol (10 : 1); IR (KBr) ν_{max} cm^{-1}: 3551, 3290, 2978, 2916, 1668, 1607, 1593; UV λ_{max} (MeOH) nm (ε): 322 (16200), 223 (17600); ^{1}H-NMR (CD_3OD) δ: 7.48 (1H, d, J=15.8 Hz, H-7), 6.89 (1H, d, J=2.0 Hz, H-2), 6.79 (1H, d, J=2.0 Hz, H-6),

* To whom correspondence should be addressed.

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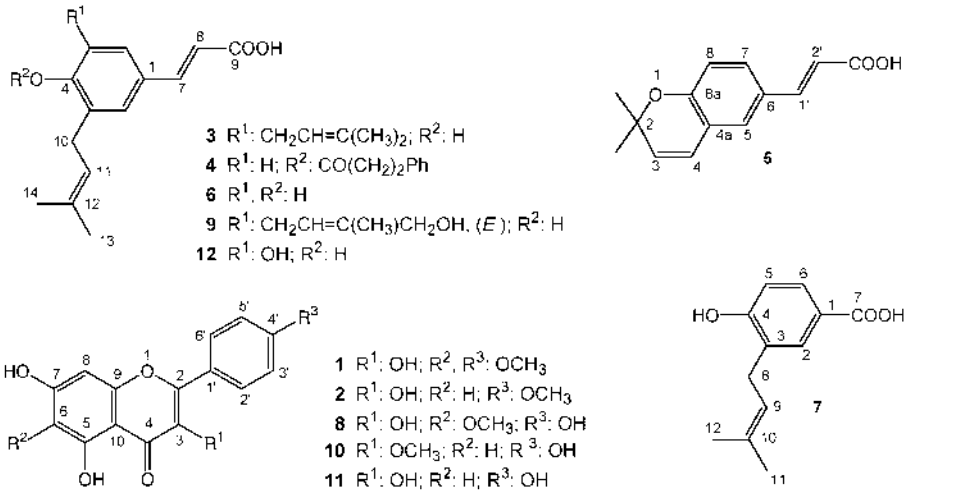

Fig. 1. Structure of Compounds Isolated from Brazilian Propolis

6.17 (1H, d, J=15.8 Hz, H-8), 5.31 (1H, m, H-11), 3.29 (2H, m, H_2-10), 1.74 (3H, s, H_3-13), 1.72 (3H, s, H_3-14); ^{13}C-NMR (CD_3OD) δ: 171.6 (C-9), 147.4 (C-4), 147.2 (C-7), 146.3 (C-3), 133.3 (C-12), 129.9 (C-5), 127.0 (C-1), 123.7 (C-11), 123.3 (C-6), 115.6 (C-8), 112.4 (C-2), 29.1 (C-10), 26.0 (C-13), 17.9 (C-14).

In the ^{1}H- and ^{13}C-NMR spectra of compound **12**, the occurrence of the following moieties was confirmed: a *trans*-cinnamic acid moiety [δ_H 7.48 (H-7), 6.89 (H-2), 6.79 (H-6), and 6.17 (H-8); δ_C 171.6 (C-9), 147.4 (C-4), 147.2 (C-7), 146.3 (C-3), 129.9 (C-5), 127.0 (C-1), 123.3 (C-6), 115.6 (C-8), and 112.4 (C-2)] and a prenyl group [δ_H 5.31 (H-11), 3.29 (H_2-10), 1.74 (H_3-13), and 1.72 (H_3-14); δ_C 133.3 (C-12), 123.7 (C-11), 29.1 (C-10), 26.0 (C-13), and 17.9 (C-14)]. The connectivities of the quaternary carbons in **12** were characterized by correlation spectroscopy *via* the HMBC spectrum (Fig. 2A). The HMBC spectrum shows that the H-2 proton and H_2-10 protons correlate with the carbon at δ_C 147.4 (C-4), suggesting that the δ_C 147.4 carbon lies at the fourth position in the aromatic ring. The observations that the H_2-10 protons correlate with the C-4 and C-5 carbons and the H-6 proton correlates with the C-10 carbon indicate that the prenyl group connects with the aromatic ring at the fifth position. These findings led us to decide the structure of compound **12** as 3-[3,4-dihydroxy-5-(3-methyl-2-butenyl)-phenyl]-2-(*E*)-propenoic acid (3,4-dihydroxy-5-prenylcinnamic acid) as shown in Fig. 1. Furthermore, the observation of NOE correlations between the protons of compound **12** (Fig. 2B) supports this structure.

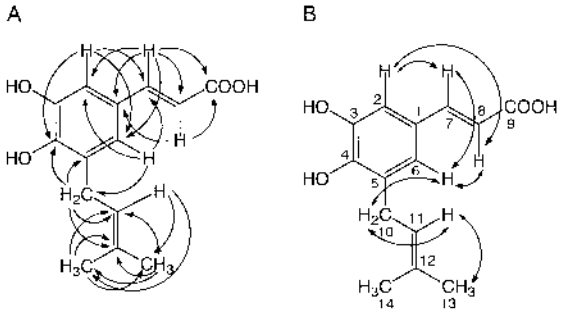

Fig. 2. NOESY and HMBC Correlations of Compound **12**

A, H→C: ^{1}H–^{13}C long-range correlations in the HMBC spectrum; B, H↔H: NOE correlations in the NOESY spectrum.

Inhibition of Peroxidation of Linoleic Acid by Compounds 1—12 The inhibitory activity against peroxidation of linoleic acid in a micelle solution was examined for compounds **1**—**12**. Table 1 summarizes their inhibitory potency. We found that compound **12** possessed the highest activity (IC_{50}, 0.17 μM) among them and was more effective than butylated hydroxytoluene (BHT; IC_{50}, 0.36 μM) under the experimental conditions employed. Among other isolated compounds, compounds **3** and **9** were also strong antioxidants, of which the antioxidative potency was first demonstrated in this study. Since compound **12** is a derivative of 4-hydroxycinnamic acid, its antioxidative activity was compared with that of various 4-hydroxycinnamic acid analogues. Table 1 also indicates that compound **12** was the strongest antioxidant among them and exhibited antioxidative potency higher than that of the well-known antioxidant ellagic acid. As to the structure–activity relationship of 4-hydroxycinnamic acid analogues, we found that the antioxidative potency was high when the hydroxyl, alkyl, or methoxy group occurs at the *ortho* position of the 4-hydroxy group. Further, the data in Table 1 indicate that 4-hydroxycinnamic acid derivatives were more effective than 4-hydroxybenzoic acid derivatives. Several authors have shown that *ortho* substitution with an electron donor, alkyl or methoxy group, increased the stability of the aryloxyl radical through electron delocalization and its antioxidative action and that further *ortho* dihydroxy substitution allowed metal chelation in addition to strong radical stabilization.[27,28] Concerning the difference between the C=C–COOH group and the COOH group, it has been proposed that the former group participates in stabilizing the aryloxyl radical by resonance.[27] Our observations support this suggestion and can explain the fact that the novel compound **12**, possessing both the electron donor *ortho* prenyl group and *ortho* dihydroxyl groups, exhibits strong antioxidative potency. Among the isolated flavonoids kaempferide **2** and kaempferol **11** were effective. Comparing the structure–activity relationship of these flavonoids, the introduction of the hydroxyl group at the third position increased the activity of radical scavenger. These results agree with those reported previously.[29,30]

Table 1. Inhibition of Peroxidation of Linoleic Acid by Antioxidative Compounds from Propolis in Comparison with 4-Hydroxycinnamic Acid and Phenolic Acid Analogues

Compounds	IC_{50} (μM)
Compounds from propolis	
1	2.2
2	0.72
3	0.44
4	160
5	56
6	2.9
7	(24 μM: 6% inhibition)[a]
8	1.4
9	0.60
10	38
11	0.92
12	0.17
4-Hydroxycinnamic acid analogues	
4-Hydroxycinnamic acid (*p*-coumaric acid)	17
4-Hydroxy-3-methoxycinnamic acid (ferulic acid)	2.1
3,5-Dimethoxy-4-hydroxycinnamic acid (sinapic acid)	0.80
3,4-Dihydroxycinnamic acid (caffeic acid)	0.41
Phenolic acid analogues	
p-Hydroxybenzoic acid	(50 μM: 15% inhibition)[a]
p-Hydroxyphenylpropionic acid	90
Protocatechuic acid	1.2
Reference samples	
n-Propylgallate	0.42
Chlorogenic acid	0.28
Ellagic acid	0.23
BHT	0.36
Quercetin	0.68

Inhibitory activity against peroxidation of linoleic acid is given. Experimental conditions are described in the text. *a*) The percent of lipid peroxidation inhibition at the described condition is shown because of the weak inhibition.

Compounds **1**—**6** were present in abundance in the Brazilian propolis [content %: **1** and **2** (combined), 0.9%; **3**, 3.5%; **4**, 0.4%; **5**, 0.4%; **6**, 1.4%, calculated by HPLC analysis]. While the other isolated constituents were small in amount (content of those constituents was less than 0.1%.). Among the isolated prenylcinnamic acid derivatives, **3**—**6** and the 3-methyl ether of **12** were previously reported to occur in different Baccharis species of Compositae that grow in tropical South American regions.[31—33] Recently it was proposed that Baccharis sp. are probably one of the main plant sources of the Brazilian propolis,[12,13,15] and our findings support this proposition. To our knowledge, the isolation of compounds **7**—**10** from Brazilian propolis reported herein is the first instance of isolation from this source.

The present results clearly demonstrate that 3,4-dihydroxy-5-prenylcinnamic acid **12** isolated from Brazilian propolis is a novel potent inhibitor of lipid peroxidation. With respect to the constituents of propolis, it seems that 4-hydroxy-3-prenylcinnamic acid derivatives are more abundant in Brazilian than in European propolis.[12,13] Among them, 3,5-diprenyl-4-hydroxycinnamic acid **3** is one of the major antioxidants in Brazilian propolis. This compound is well known to possess antibacterial,[10] choleretic,[19,20] and antitumor[34] properties. Since the structure of compound **12** is similar to that of compound **3**, the former is an attractive material with respect to curing and preventing diseases mediated by free radicals. Further study on its practical application to medical and biological fields is being done in our laboratory.

Experimental

Materials Brazilian propolis was purchased from MN EXPORTAÇÃO E REPRESENTAÇÃO LTDA in Brazil. Kaempferide, kaempferol, and quercetin were obtained from Extrasynthese S.A., Genay, France; AAPH, BHT, *p*-coumaric acid, and other phenolic acids, from Wako Pure Chemicals, Osaka. Lubrol PX came from Nacalai Tesque, Kyoto; and linoleic acid from Sigma Chemicals, St. Louis, MO.

For column chromatographies, Iatrobeads-80100, 75—150 μm (for silica gel column A) was purchased from Iatron Laboratories,Tokyo; and Silica gel 60 No.7734, 63—200 μm (for silica gel column B), from E. Merck, Darmstadt, Germany. Silica gel BW-127ZH, 53—150 μm (for silica gel column C), Microbeads MB-4B, 75—150 μm (for silica gel column D), Chromatorex DMS DM1020, 75—150 μm (for DMS column), Chromatorex ODS DM1020T, 75—150 μm and Chromatorex DM2035MT, 44—75 μm (for ODS column A and B, respectively) were obtained from Fuji Silysia Chemicals, Kasugai, Japan. Sephadex LH-20, 27—163 μm, was from Amersham Pharmacia Biotech, Uppsala, Sweden.

Silica gel 60 F_{254} precoated plate No. 5642 and RP-18W F_{254S} precoated plate No.13124 for HPTLC, and Silica gel 60 F_{254} precoated plate No.13895 for preparative TLC were obtained from E. Merck.

Instruments for Chemical Identification The following instruments were used to obtain physical data: melting points, Yanagimoto micro-melting point apparatus MP-S3 (Yanagimoto Shoji, Kyoto); IR spectra, Hitachi 270-30 infrared spectrophotometer (Hitachi, Tokyo) or Shimadzu FT-IR 8300 infrared spectrophotometer (Shimadzu, Kyoto); UV spectra, Shimadzu MPS-2000 spectrophotometer (Shimadzu); NMR spectra, JEOL EX-270 specrtometer (270 MHz for ^{1}H-NMR and 68 MHz for ^{13}C-NMR, JEOL, Tokyo); MS spectra, JEOL-Mstation JMS700 spectrometer (JEOL); HPLC spectra, LC-4A liquid chromatograph equipped with SPD-2AS UV detector (Shimadzu). Separation by HPLC was performed on a reverse-phase TSKgel ODS-80TM column (15 cm×4.6 mm i.d., 5 μm, Tosoh, Tokyo) with water–methanol–acetic acid (32 : 65 : 3) as a mobile phase at a flow rate of 1 ml/min at 36 °C, and absorbancy of the eluate was monitored at 275 nm. For HPTLC, detection was achieved by illumination with a UV lamp at 254 nm.

Isolation of Constituents of Propolis Propolis (216 g) was extracted with 70% ethanol (3.2 l), and the extract was concentrated *in vacuo*. The residue (103 g) was suspended in water and extracted with ether (4 l×3) to give an ether extract (71 g). The ether extract was chromatographed on a silica gel column A with chloroform and then with chloroform–methanol (60 : 1—10 : 1) to give 3 fractions (Fr. 1—Fr. 3).

Fraction 1 (30 g) was rechromatographed on a silica gel column B with chloroform and then chloroform–methanol (40 : 1—10 : 1) to give 3 fractions (Fr. 1-1, Fr. 1-2, and Fr. 1-3). Fraction 1-1 (4.3 g) was purified by silica gel column B chromatography with chloroform–methanol (50 : 1—10 : 1) and washing with chloroform–methanol (3 : 1) to give compound **1** (90 mg). Fraction 1-2 (10 g) was chromatographed on a silica gel column B with chloroform–methanol (60 : 1—10 : 1) to give Fr. 1-2-1 and Fr. 1-2-2. Fraction 1-2-1 (1.1 g) was washed with chloroform–methanol (100 : 1) to give compound **2** (120 mg). Fraction 1-2-2 (2.0 g) was purified by ODS column A chromatography with 80% methanol to give compound **3** (1.3 g). Fraction 1-3 (2.1 g) was successively subjected to silica gel column C chromatography with chloroform–methanol (20 : 1) and DMS column chromatography with 60—80% methanol to give Fr. 1-3-1 and Fr. 1-3-2. Fraction 1-3-1 (430 mg) was applied onto a DMS column with 65—70% methanol, and then twice onto an ODS column A with 80% methanol to give compound **4** (87 mg). Repeated ODS column A chromatography of Fr. 1-3-2 (410 mg) using 75% methanol afforded compound **5** (170 mg).

Fraction 2 (14 g) was chromatographed on a silica gel column C with chloroform–methanol (50 : 1—10 : 1) to give Fr. 2-1 and Fr. 2-2. Purification of Fr. 2-1 (3.7 g) was carried out by silica gel column B, DMS column, and ODS column A chromatographies in a similar way to give compound **6** (220 mg). Fraction 2-2 (0.6 g) was chromatographed on an ODS column A with 65% methanol to give Fr. 2-2-1 and Fr. 2-2-2. Fraction 2-2-1 (83 mg) was successively subjected to silica gel column D chromatography with chloroform–methanol (30 : 1), preparative TLC with chloroform–methanol (10 : 1), and ODS column B chromatography with 65% methanol to give compound **7** (28 mg). Washing Fr. 2-2-2 (68 mg) with methanol provided compound **8** (15 mg).

Fraction 3 (17 g) was chromatographed on a silica gel column C with chloroform–methanol (40 : 1—10 : 1) to give Fr. 3-1 and Fr. 3-2. Fraction 3-1 (5.0 g) was subjected to DMS column chromatography with 55—90% methanol to give Fr. 3-1-1 and Fr. 3-1-2. Fraction 3-1-1 (500 mg) was successively subjected to ODS column A chromatography and Sephadex LH-20 column chromatography with 70% methanol to give **9** (54 mg). Fraction 3-1-2 (250 mg) was purified in the same manner as for compound **9** to give compounds **10** (18 mg) and **11** (7 mg). Fraction 3-2 (2.8 g) was successively subjected to DMS column chromatography with 55—90% methanol, ODS column A chromatography with 70% methanol, silica gel column B chromatography with chloroform–methanol (20 : 1—5 : 1), and Sephadex LH-20 column chromatography with 70% methanol to afford compound **12** (24 mg).

Among the 11 known compounds, detailed data of compounds **7** and **10** were obtained first and are described here. Physical data of the new compound **12** were described in Results and Discussion.

Compound **7**: 4-Hydroxy-3-prenylbenzoic Acid[23]: Colorless amorphous solid, mp 99—100 °C. HR-FAB-MS (positive mode) *m/z*: 207.1035 $(M+H)^+$ (Calcd for $C_{12}H_{15}O_3$: 207.1021). HPTLC RP-18W F_{254S} *Rf*: 0.69, 75% methanol. IR (KBr) ν_{max} cm^{-1}: 3500—2800 (br), 1684, 1610. ^{1}H-NMR (CD_3OD) δ: 7.74 (1H, d, J=2.0 Hz, H-2), 7.71 (1H, dd, J=2.3, 8.2 Hz, H-6), 6.78 (1H, d, J=8.2 Hz, H-5), 5.3 (1H, m, H-9), 3.3 (2H, m, H_2-8), 1.75 (3H, s, H_3-11), 1.72 (3H, s, H_3-12). ^{13}C-NMR (CD_3OD) δ: 170.5 (C-7), 161.1 (C-4), 133.7 (C-10), 132.6 (C-2), 130.5 (C-6), 129.3 (C-3), 123.3 (C-9), 122.6 (C-1), 115.3 (C-5), 29.1 (C-8), 26.0 (C-11), 17.9 (C-12).

Compound **10**: 3-Methylkaempferol[26]: Yellow needles, mp 283—287 °C. HR-FAB-MS (positive mode) *m/z*: 301.0712 $(M+H)^+$ (Calcd for $C_{16}H_{13}O_6$: 301.0712). HPTLC Silica gel 60 F_{254} *Rf*: 0.50, chloroform–methanol (10 : 1). IR (KBr) ν_{max} cm^{-1}: 3500—3000 (br), 1660, 1615. ^{1}H-NMR (DMSO-d_6) δ: 12.7 (1H, br s, OH-5), 7.94 (2H, d, J=8.6 Hz, H-2′,6′), 6.95 (2H, d, J=8.9 Hz, H-3′,5′), 6.44 (1H, d, J=2.0 Hz, H-8), 6.20 (1H, d, J=2.0 Hz, H-6), 3.78 (3H, s, H_3-11). ^{13}C-NMR (DMSO-d_6) δ: 177.8 (C-4), 164.1 (C-7), 161.2 (C-5), 160.1 (C-4′), 156.3 (C-9), 155.5 (C-2), 137.5 (C-3), 130.0 (C-2′, 6′), 120.5 (C-1′), 115.5 (C-3′, 5′), 104.1 (C-10), 98.5 (C-6), 93.6 (C-8), 59.6 (C-11).

Inhibition of Peroxidation of Linoleic Acid Linoleic acid micelle solution was prepared by adding 0.35 ml of 600 mM linoleic acid in ethanol to 62 ml of 0.3% Lubrol PX-20 mM sodium phosphate buffer (pH 7.4) and then sonicating the mixture for 3 min. The reaction mixture (1 ml) contained 0.89 ml of linoleic acid micelle solution, 0.01 ml of a test sample dissolved in dimethylsulfoxide, and 0.1 ml of 5 mM AAPH in 0.3% Lubrol PX-20 mM sodium phosphate buffer (pH 7.4). The final concentration of each component was as follows : linoleic acid, 3 mM; AAPH, 0.5 mM; dimethylsulfoxide, 1%; and ethanol, 0.5%. After the mixture had been incubated at 37 °C for 1 h, the reaction was stopped by the addition of 1 ml of methanol; and then 1 ml of the mixture was mixed with 4 ml of 70% methanol. Amounts of linoleic acid hydroperoxide were measured in terms of their conjugated diene with a UV spectrophotometer at 234 nm. As a control, 0.01 ml of dimethylsulfoxide was used.

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