Antioxidative Components Isolated from the Seed of Tamarind (*Tamarindus indica* L.)

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Antioxidative activity of tamarind (*Tamarindus indica* L.) seeds was investigated. An ethanol extract prepared from the seed coat exhibited antioxidative activity as measured by the thiocyanate and thiobarbituric acid (TBA) method, but there was no activity in the extract prepared from germ. The ethyl acetate extract prepared from seed coat had strong antioxidative activity. To determine antioxidative compounds in the seed coat chemically, preparative HPLC was carried out, then 2-hydroxy-3′,4′-dihydroxyacetophenone (TA0), methyl 3,4-dihydroxybenzoate (TA1), 3,4-dihydroxyphenyl acetate (TA2), and (−)-epicatechin (TA3) were isolated from the ethyl acetate extract and identified. TA0, TA1, and TA2 had strong antioxidative activity in the linoleic acid autoxidation system as measured by the thiocyanate and TBA method as well as α-tocopherol. These results suggest that tamarind seed coat, a byproduct of tamarind gum industries, can be used as a safe and low-cost source of antioxidants.

Keywords: *Antioxidative activity; tamarind; seed coat; phenolic derivatives*

Lipid peroxidation is known as one of the major factors in deterioration during the storage and processing of food. In addition, it is thought that lipid peroxidation is strongly associated with carcinogenesis, mutagenesis, aging, and atherosclerosis (Yagi, 1987; Cutlar, 1984, 1992). The addition of antioxidants has become popular as a means of increasing the shelf life of food products and improving the stability of lipids and lipid-containing foods by preventing loss of sensory and nutritional quality. In living systems, dietary antioxidants such as β-carotene, α-tocopherol, and ascorbic acid may be effective in protection from oxidative damage as well as in enzymatic protection by endogenous enzymes such as superoxide dismutase, glutathione peroxidase, and catalase.

Effective synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been used in food industries; however, these synthetic antioxidants are suspected to be carcinogenic (Branen, 1975; Ito, 1983). Tocopherol, a natural antioxidant, is less effective than synthetic ones and the manufacturing cost is high.

Recently, one of the several natural antioxidants such as polyphenol or β-diketone type containing in dietary plants reported to play an important role in prevention of carcinogenesis and to extend life span in animals and dietary antioxidants was reported to offer effective protection against peroxidative damages in living systems (Cutlar, 1984; Osawa et al., 1990; Hirose et al., 1994). Therefore, much attention has been focused on natural antioxidants, and some polyphenol and β-diketone type of antioxidants isolated from natural sources with high antioxidative activity have been reported (Tsuda et al., 1994; Okamura et al., 1993; Katsuzaki et al., 1993; Osawa et al., 1992; Nishina et al., 1991).

Tamarind (*Tamaridus indica* L.) belongs to Leguminosae, and the place of its origin is said to be Africa. It grows naturally in tropical and subtropical regions now and is one of the most important plant resources as food materials. The pulp is used in spices and seasoning, and it is accepted as a herb medicine in parts of the world. The flower and leaf are eaten as vegetables. The germ obtained from the seeds is used for manufacturing tamarind gum, and it has been added to many kinds of foods in Japan to improve their viscosity. However, the seed coat as byproduct of tamarind gum has been hardly used, and there has been no attention to the seeds from the viewpoint of antioxidative activity. This background prompted us to investigate antioxidants in the seeds of tamarind chemically. We have already reported the antioxidative activity of tamarind seeds (Tsuda et al., 1993). However, the antioxidants contained in the seeds are still unidentified. This paper describes how antioxidants were isolated and identified from tamarind seeds and the antioxidative activity in a model system was examined.

MATERIALS AND METHODS

Tamarind seeds and tamarind seed coat were obtained from Yaegaki Zymotechnics, Inc., Japan. The seeds were cultivated in India in 1992, cleaned, and stored at 4 °C until used. Linoleic acid and α-tocopherol were obtained from Wako Pure Chemical Industries, Ltd., Japan. An authentic sample of (−)-epicatechin was purchased from Aldrich Chemical Co.

Extraction of Antioxidants from Tamarind Seeds. The seed coats and germs were removed manually, and the separated seed coats and germs were ground by using a chemical grinder (NRK Inc., Japan) and extracted three times with ethanol and then filtered. The filtrates were concentrated to dryness *in vacuo* to obtain crude seed coat extracts and germ extracts.

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0021-8561/94/1442-2671$04.50/0

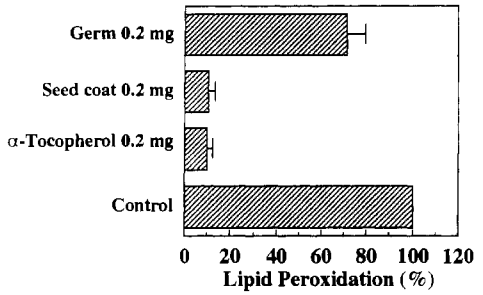

Figure 1. Antioxidative activity of crude extracts prepared from tamarind seed coats, germs, and α-tocopherol (200 μg) as measured by the thiocyanate method. Reported values are mean ± SD (n = 3). A control containing no added samples or standard on its value represents 100% lipid peroxidation.

Extraction of Antioxidants from Seed Coats with Four Solvents. The four batches of ground seed coats were separately extracted for 17 h three times with four solvents (ethyl acetate, 1:1 ethyl acetate/ethanol, ethanol, and methanol), and then four crude extracts (ethyl acetate extract, 1:1 ethyl acetate/ethanol extract, ethanol extract, methanol extract) were obtained by the identical procedure described above.

Antioxidative Assay for Crude Extracts. Antioxidative activity was carried out by using the linoleic acid system (Osawa and Namiki, 1981). Each sample (200 μg) was added to a solution mixture of linoleic acid (0.13 mL), 99.0% distilled ethanol (10 mL), and 50 mM phosphate buffer (pH 7.0, 10 mL); the total volume was adjusted to 25 mL with distilled water. The solution was incubated at 40 °C, and the degree of oxidation was measured according to the thiocyanate method (Mitsuda et al., 1966) for measuring peroxides by reading the absorbance at 500 nm after coloring with $FeCl_2$ and ammonium thiocyanate and by the thiobarbituric acid (TBA) method (Ottolenghi, 1959). α-Tocopherol (200 μg) was used as standard sample. Statistical analysis was performed by using Student's t-test.

Isolation of Antioxidative Compounds from Tamarind Seed Coat. The ground seed coat (460 g) was extracted with 2 L of ethyl acetate four times to obtain crude seed coat extract (3.4 g). Preparative high-performance liquid chromatography (HPLC) was carried out by using a Develosil ODS-10 column (Nomura Chemical Co. Ltd., Japan, 20 mm × 250 mm) with a spectrophotometric detector (Shimadzu Works, Co. Ltd., LC-10AV) with UV (280 nm) and 0.1% trifluoroacetic acid (TFA)/17% acetonitrile as a solvent at flow rate of 5.0 mL/min. TA0 (7.8 mg), TA1 (21.3 mg), TA2 (281.3 mg), and TA3 (47.0 mg) were obtained.

Instrumental Analysis of Isolated Compounds. UV-absorption spectra were recorded on a spectrophotometer with a JASCO U best-50. The samples were dissolved in methanol and measured. IR spectra were recorded on a JASCO FT/IR-8000 with KBr. The electron impact mass spectra (EI-MS) were recorded on a JEOL JMS-DX-705L. ^{1}H-NMR and ^{13}C-NMR spectra were obtained by a Bruker ARX-400 NMR instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C) in d-acetone containing tetramethylsilane (TMS) as an internal standard for all samples.

Antioxidative Assay for Isolated Compounds. Antioxidative activity of the isolated compounds was determined by the thiocyanate and TBA method with the linoleic acid system described above. The concentration of the compounds was 20 μM in each case. α-Tocopherol (20 μM) was also used as a positive control. Statistical analysis was performed by using Student's t-test.

RESULTS AND DISCUSSION

Antioxidative Activity of Crude Extracts. Antioxidative activity of crude extracts from the seed coat and germ as measured by the thiocyanate method are shown in Figure 1. There was weak antioxidative activity in the extract prepared from germ. On the other hand, seed coat extract exhibited strong antioxidative activity. The extent of activity was the same as that of 200 μg of α-tocopherol (not significant). The results measured by the TBA method showed the same tendency (data not shown). These results suggest that the antioxidative components are present in seed coat, not germ. Then the suitable extracting solvent was examined from four organic solvents.

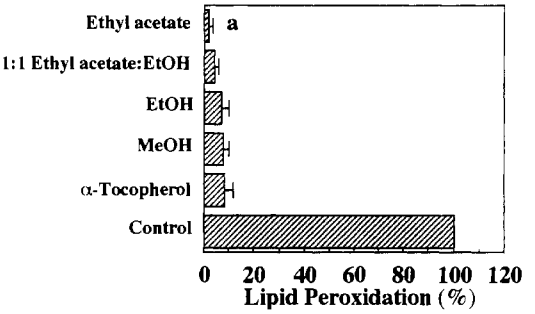

Figure 2. Antioxidative activity of crude samples (200 μg) extracted with four solvents from tamarind seed coats as measured by the thiocyanate method. α-Tocopherol (200 μg) was used for the standard sample. Reported values are mean ± SD (n = 3). A control containing no added samples or standard on its value represents 100% lipid peroxidation. (a) $P < 0.05$, compared with α-tocopherol.

Antioxidative activity of the four solvent extracts prepared from the seed coat as measured by the thiocyanate method is shown in Figure 2. All four extracts had strong antioxidative activity. One of the extracts, ethyl acetate extract showed the strongest antioxidative activity, and the extent of the activity was stronger than α-tocopherol ($P < 0.05$). Therefore, we focused on ethyl acetate extract, and the isolation and characterization of antioxidative compounds extracted from the seed coat by ethyl acetate were carried out.

Isolation and Identification of Antioxidative Compounds. Ethyl acetate extract prepared from the seed coat was purified by using preparative HPLC technique, and four compounds (TA0, TA1, TA2, and TA3) were obtained.

Identification of TA0 (2-Hydroxy-3′,4′-dihydroxyacetophenone). TA0 exhibited the following properties: UV λ_{max} in methanol (nm) 309.0 (log ϵ = 3.88), 277.0 (log ϵ = 3.99), 231.0 (log ϵ = 4.18); IR υ_{max} in KBr (cm^{-1}) 3125, 1669, 1607, 1522, 1422, 1196, 1006, 775; EI-MS (m/z) 168 [M]$^+$; ^{1}H NMR (in d-acetone) δ 7.31 (1H, dd, J = 8.3, 1.9, H6), 7.29 (1H, d, J = 2.0, H2), 6.82 (1H, d, J = 8.0, H5), 4.73 (3H, s, CH_2); ^{13}C NMR (in d-acetone) δ 198.7 (s, C=O), 151.7 (s, C4), 145.7 (s, C3), 127.0 (s, C1), 122.3 (d, C6), 116.1 (d, C5), 115.2 (d, C2), 65.3 (t, CH_2). Given these results and considering two-dimensional NMR spectra, ^{1}H−^{1}H COSY, HMQC, and HMBC (data not shown), TA0 was identified as 2-hydroxy-3′,4′-dihydroxyacetophenone (Andersen, 1971; Andersen and Barrett, 1971; Barrett, 1977). This compound was isolated from insect hard cuticle, but the antioxidative activity has not been reported.

Identification of TA1 (Methyl 3,4-Dihydroxybenzoate). TA1 exhibited the following properties: UV λ_{max} in methanol (nm) 295.0 (log ϵ = 3.50), 259.5 (log ϵ = 3.76); IR υ_{max} in KBr (cm^{-1}) 3316, 1663, 1601, 1526, 1433, 1293, 766; EI-MS (m/z) 168 [M]$^+$; ^{1}H NMR (in d-acetone) δ 7.44 (1H, d, J = 1.9, H2), 7.42 (1H, dd, J = 8.3, 2.0, H6), 6.80 (1H, d, J = 8.0, H5), 3.35 (3H, s, OCH_3); ^{13}C NMR (in d-acetone) δ 170.2 (s, C=O), 151.5 (s, C4), 146.0 (s, C3), 123.9 (d, C6), 123.1 (s, C1), 117.7 (d, C2), 115.6 (d, C5), 49.8 (q, OCH_3). From these results and two-

dimensional NMR spectra data, $^1H-^1H$ COSY, HMQC, and HMBC (data not shown), TA1 was identified as methyl 3,4-dihydroxybenzoate (Yun-Choi et al., 1987).

Identification of TA2 (3,4-Dihydroxyphenyl Acetate). TA2 gave the most yield for four isolated compounds and exhibited the following properties: UV λ_{max} in methanol (nm) 290.0 (log ϵ = 3.30), 260.0 (log ϵ = 3.54); IR υ_{max} in KBr (cm^{-1}) 3256, 1676, 1603, 1528,1428, 1304, 766; EI-MS (*m/z*) 168 $[M]^+$; ^1H NMR (in *d*-acetone) δ 7.55 (1H, d, J = 1.9, H2), 7.50 (1H, dd, J = 8.3, 2.0, H6), 6.80 (1H, d, J = 8.2, H5), 2.07 (3H, s, CH_3); ^{13}C NMR (in *d*-acetone) δ 168.6 (s, C=O), 150.8 (s, C4), 145.4 (s, C3), 123.8 (d, C6), 122.7 (s, C1), 117.5 (d, C2), 115.7 (d, C5), 30.6 (q, CH_3). From these spectral data and two-dimensional NMR spectral data, $^1H-^1H$ COSY, HMQC, and HMBC (data not shown), TA2 was determined to be 3,4-dihydroxyphenyl acetate. This is the first case that isolation from plant sources and examination of antioxidative activity have been reported.

Identification of TA3 [(−)-Epicatechin]. TA3 exhibited the following properties: UV λ_{max} in methanol (nm): 280.5 (log ϵ = 3.42); IR υ_{max} in KBr (cm^{-1}) 3360, 1688, 1607, 1520, 1468, 1367, 1144, 795; EI-MS (*m/z*) 290 $[M]^+$; ^1H NMR (in *d*-acetone) δ 7.03 (1H, d, J = 1.8, H2′), 6.82 (1H, dd, J = 8.3, 1.8, H6′), 6.77 (1H, d, J = 8.2, H5′), 6.00 (1H, d, J = 2.3, H8), 5.90 (1H, d, J = 2.3, H6), 4.86 (1H, s, H2), 4.19 (1H, ddd, J = 4.5, 3.3, 1.6, H3), 2.85 (1H, dd, J = 16.6, 4.5, H4), 2.72 (1H, dd, J = 16.6, 3.2, H4); ^{13}C NMR (in *d*-acetone) δ 157.5 (s, C9), 157.4 (s, C7), 157.0 (s, C5), 145.3 (d, C4′), 145.1 (s, C3′), 132.1 (s, C1′), 119.2 (d, C6′), 115.4 (d, C5′), 115.1 (d, C2′), 99.7 (s, C10), 96.1 (d, C6), 95.6 (d, C8′), 79.3 (d, C2), 66.8 (d, C3), 28.6 (t, C4). Considering these spectral data and two-dimensional NMR spectra, $^1H-^1H$ COSY, HMQC, and HMBC (data not shown), TA3 could be identified tentatively as (−)-epicatechin. To confirm this, instrumental analysis of authentic (−)-epicatechin performed and compared with the data of TA3. The NMR data of authentic samples exhibit as follows: ^1H NMR (in *d*-acetone) δ 7.07 (1H, d, J = 1.9, H2′), 6.86 (1H, dd, J = 8.3, 1.8, H6′), 6.80 (1H, d, J = 8.3, H5′), 6.04 (1H, d, J = 2.3, H8), 5.94 (1H, d, J = 2.3, H6), 4.90 (1H, s, H2), 4.22 (1H, ddd, J = 4.5, 3.3, 1.6, H3), 2.88 (1H, dd, J = 16.6, 4.5, H4), 2.76 (1H, dd, J = 16.6, 3.2, H4); ^{13}C NMR (in *d*-acetone) δ 157.5 (s, C9), 157.4 (s, C7), 157.0 (s, C5), 145.2 (d, C4′), 145.1 (s, C3′), 132.1 (s, C1′), 119.2 (d, C6′), 115.4 (d, C5′), 115.1 (d, C2′), 99.7 (s, C10), 96.0 (d, C6), 95.6 (d, C8′), 79.3 (d, C2), 66.8 (d, C3), 28.9 (t, C4). These authentic data and UV and IR spectral data (data not shown) were in agreement with those of TA3. The instrumental data obtained by Foo and Karchesy (1989) and Cui et al. (1992) on (−)-epicatechin are also consistent with our instrumental data. Therefore, TA3 was identified as (−)-epicatechin. The chemical structures of these isolated compounds are elucidated in Figure 3.

Antioxidative Activity of Isolated Compounds. Antioxidative activities of isolated compounds of TA0, TA1, TA2, and TA3 as measured by the TBA method are shown in Figure 4. All four compounds had antioxidative activities in the model system. In these antioxidants, the dihydroxy type of benzene derivatives (TA0, TA1, and TA2) had stronger antioxidative activity than TA3. The extents of the activity of these compounds (TA0, TA1, and TA2) were the same as α-tocopherol (not significant); TA3 had weaker activity than

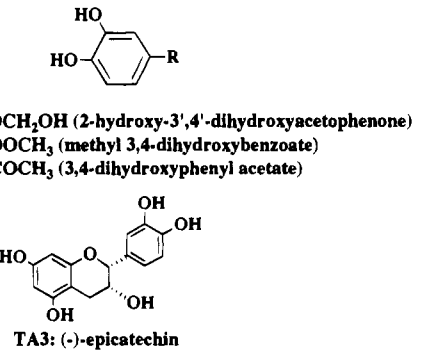

Figure 3. Chemical structures of antioxidative compounds isolated from tamarind seed coat.

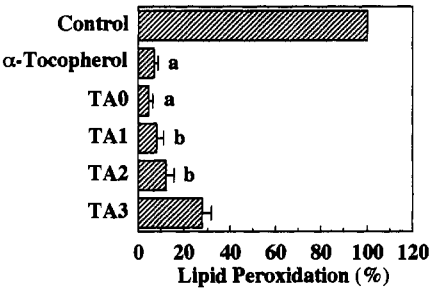

Figure 4. Antioxidative activity of isolated compounds (20 μM) from tamarind seed coat as measured by the thiocyanate method. α-Tocopherol (20 μM) was used as standard sample. Reported values are mean ± SD (n = 3). A control containing no added samples or standard on its value represents 100% lipid peroxidation. TA0, 2-hydroxy-3′,4′-dihydroxyacetophenone; TA1, methyl 3,4-dihydroxybenzoate; TA2, 3,4-dihydroxyphenyl acetate; TA3, (−)-epicatechin. (a) P < 0.001, compared with TA3; (b) P < 0.01, compared with TA3.

α-tocopherol. The results as measured by the thiocyanate method showed the same tendency (data not shown).

Conclusion. Tamarind is one of the most important plant resources for food material in tropical region. The seeds are used for extracting tamarind gum, and the seed coats are a byproduct of manufacturing tamarind gum; therefore, we gave attention to it as a safe and low-cost source of antioxidants and isolated and identified four antioxidative compounds. These antioxidants may be used not only for increasing the shelf life of foods by preventing lipid peroxidation but also for protecting oxidative damage in living systems by scavenging active oxygen radicals. Further investigation is necessary of these antioxidants in vitro and in vivo.

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Received for review June 9, 1994. Revised manuscript received September 23, 1994. Accepted October 4, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1994.