Extraction and Identification of Antioxidant Components from the Leaves of Mulberry (*Morus alba* L.)

Gow-Chin Yen,* She-Ching Wu, and Pin-Der Duh

Department of Food Science, National Chung Hsing University, 250 Kuokuang Road, Taichung, Taiwan, Republic of China

The antioxidant activity of mulberry leaves (*Morus alba* var. Acidosa G. cv. Taisang 2) was investigated. The results indicated that methanolic extracts of mulberry leaves (MEML) showed stronger antioxidant activity and that methanol gave higher yields of extract than other organic solvents. The MEML exhibited 78.2% inhibition on peroxidation of linoleic acid, greater than that of α-tocopherol (72.1%) but equal to that of butylated hydroxyanisole. The MEML were separated into nine fractioins by thin-layer chromatography (TLC). Two of the fractions [R_f = 0.92 (I) and R_f = 0.68 (II)] possessed remarkable antioxidant activities. These two fractions showed 77.3 and 72.0% inhibition on peroxidation of linoleic acid, respectively. Fraction I (R_f = 0.92) was further purified again with TLC; the stronger antioxidant activity was found in subfraction Ia (R_f = 0.87), while fraction II (R_f = 0.68) was not fractionated into any subfractions. On the basis of UV−vis spectral, HPLC, NMR, and MS data, the antioxidant components of Ia and II were identified as β-carotene and α-tocopherol, respectively.

Keywords: *Mulberry leaves; antioxidant; β-carotene; α-tocopherol*

INTRODUCTION

Lipid peroxidation in fats and fatty foods not only brings about chemical spoilage in foods but also produces free radicals or active oxygens such as peroxyl and hydroxyl radicals, which are purportedly associated with carcinogenesis, mutagenesis, and aging (Tagi, 1987). The most widely used synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are suspected of causing some safety concerns (Branen, 1975; Imaida et al., 1983). Therefore, these antioxidants have been restricted recently, although they have marked antioxidant activity. α-Tocopherol, a natural antioxidant, is an effective antioxidant for lipid-containing foods but has limited usage (Osawa and Namiki, 1981). The fact that various antioxidants occur naturally in plants has been proved (Larson, 1988; Ramarathnam et al., 1988). Therefore, identification and development of safer, natural antioxidants is beneficial.

Mulberry (*Morus alba* L.) leaves containing many nutritional components are the best feed for silkworms. Traditionally, mulberry leaves are used as a medicinal herb in Chinese culture, but no investigation on antioxidant activity has been reported so far. Therefore, the purpose of this study was to investigate the antioxidant activity of various organic solvent extracts and to identify the major antioxidative components of mulberry leaves.

MATERIALS AND METHODS

Material. Mulberry (*M. alba* L. var. Acidosa G. cv. Taisang 2) leaves were obtained from The Taiwan Agricultural and Sericultural Experiment Station, Kung-Kuan, Miaoli, Taiwan. The leaves were harvested, cleaned, freeze-dried, and ground into a fine powder in a mill (Tecator Cemotec 1090 sample mill, Hoganas, Sweden). The material that passed through an 80-mesh sieve was retained for use, sealed in a plastic bottle, and stored at 4 °C until used. Soybean oil obtained from Chia Shin Co. (Taichung, Taiwan) was filtered with active carbon (Sigma Chemical Co., St. Louis, MO) to remove tocopherol (Chang et al., 1983) and stored in a refrigerator.

Chemicals. Ammonium thiocyanate, ferrous chloride tetrahydrate, sodium dihydrogen phosphate, and disodium hydrogen phosphate were purchased from E. Merck (Darmstadt, Germany). Linoleic acid, sodium carbonate, BHA, β-carotene, and α-tocopherol were obtained from Sigma.

Chemical Analysis. The percentages of moisture, crude protein, crude fat, crude fiber, and ash in mulberry leaves were determined according to AOAC (1984) Methods 14.062, 14.067, 14.060, 14.064, and 14.063, respectively.

Extraction of Antioxidant Components from Mulberry Leaves. A 10 g of sample of each mulberry leave powder was extracted for 24 h with 100 mL each of *n*-hexane, ethyl acetate, acetone, or methanol, respectively, in a shaking incubator at room temperature. The extracts were filtered with Whatman No. 1 filter paper. The filtrates were evaporated *in vacuo* and weighed to determine the yield of soluble constituents.

Thin-Layer Chromatography (TLC). An aliquot of methanolic extract (10 μL) was spotted on a precoated silica gel plate (5 × 20 cm, Kieselgel 60 F, 0.25 mm, Merck), which had been activated for 30 min at 100 °C. The plate was developed in the ascending direction for 17.5 cm with the solvent system benzene/ethyl formate/formic acid (75:24:1 v/v/v) (BEF). After drying of the plate, the spots were located by a TLC UV scanner (CAMAG Ltd., Muttenz, Switzerland) at 254 nm. Additionally, according to the method of Chang et al. (1983), the plate developed with BEF solvent was dipped in a freshly prepared soybean oil/*n*-hexane (97:3 v/v) solution for 10 s. Then, the plate was immediately placed in the UV cabinet at 254 nm. The time of disappearance of each fluorescent spot was recorded for comparison with fluorescence-persisting time (FPT) for antioxidant activity evaluation.

Purification of the Active Fractions. To purify and obtain sufficient quantities of the antioxidative components in mulberry leaves, the methanolic extracts (1.0 mL) were streaked on a precoated silica gel plate (20 × 20 cm, Art. 13894, Kieselgel 60, 0.5 mm, Merck) and developed with BEF (75:24:1 v/v/v). Immediately the fractions with long FPT were scraped. Each fraction was isolated with methanol; then the extracts were filtered through a 0.45 μm Millipore (Bedford, MA) filter and evaporated to dryness *in vacuo* below 40 °C.

* Author to whom correspondence should be addressed (fax 886-4-261-2962).

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Table 1. Proximate Analysis of Mulberry Leaves[a]

	%		%
moisture	6.1	ash	11.3
crude protein	22.2	crude fiber	9.9
crude fat	6.9	N-free extract[b]	43.6

[a] Values are means of duplicate analyses. [b] The percentage of nitrogen-free extract was determined by 100% minus the total percentage of other constituents.

The residue was weighed to determine the yield of each fraction and then redissolved in methanol to determine the antioxidant activity.

The spots possessing antioxidant activity were developed on a silica gel plate with the following solvent systems: BEF; toluene/ethyl formate/formic acid (66:29:5 v/v/v) (TEF); toluene/chloroform/acetone (40:25:35 v/v/v) (TCA); benzene/glacial acetic acid (66:33 v/v) (BG); benzene/acetone (90:10 v/v) (BA); *n*-butanol/glacial acetic acid/water (60:20:20 v/v/v) (BGW); and *n*-butanol/butyl acetate/formic acid (70:20:10 v/v/v) (BBF).

Antioxidant Activity Determination. The antioxidant activity of all organic solvent extracts and separated spots was determined according to the thiocyanate method (Mitsuda et al., 1966). Each sample (1.0 mg/0.5 mL) was added to a solution mixture of linoleic acid (2.5 mL, 0.02 M) and potassium phosphate buffer (2 mL, 0.2 M, pH 7.0). The mixed solution, in a conical flask, was incubated at 37 °C. At regular intervals, the peroxide value was determined by reading the absorbance at 500 nm, after reaction with $FeCl_2$ and thiocyanate. The solutions without added organic solvent extracts were as blank samples. All test data are average of triplicate analyses.

High-Performance Liquid Chromatography (HPLC). HPLC analysis was performed with a Hitachi liquid chromatograph (Hitachi, Ltd., Tokyo, Japan), consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe-loading sample injector, and a Model D-2000 integrator. The other HPLC analytical conditions are as follows: [condition A (Kitada et al., 1989)] LiChrospher RP-18 reversed-phase column (5 μm, 250 × 4 mm i.d., Merck), eluting solvent of acetone/methanol/acetonitrile (1:2:2 v/v/v) at a flow rate of 0.7 mL/min, monitor UV−vis detector (L-4200) at 470 nm; [condition B (Carpenter, 1979)] LiChrosorb Si-60 (7 μm, 250 × 4 mm i.d., Merck), eluting solvent of *n*-hexane/2-propanol/ethanol (100:0.3:0.2 v/v/v) at a flow rate of 0.7 mL/min, monitor UV detector (L-4200) at 295 nm.

UV−Vis Spectrometry. UV−vis absorption spectra of the active components in methanol were recorded on a Hitachi U-2000 spectrophotometer.

Nuclear Magnetic Resonance (NMR) Spectrometry. NMR spectra were recorded with a Varian VXR-300S FT-NMR spectrometer operating at 299.95 MHz for ^1H NMR and at 75.43 MHz for ^{13}C NMR with complete proton decoupling. The spectra were observed in $CDCl_3$. The sweep widths, pulse angles, and repetition rates for ^1H NMR were 5500.0 Hz, 7.0 μs, and 0 s, respectively, and for ^{13}C NMR were 22000.0 Hz, 7.0 μs, and 2.0 s, respectively. The chemical shifts are reported in parts per million values from tetramethylsilane.

Mass Spectrometry. Mass spectra of active components were recorded using an electron ionization (EI) mode at 70 eV with a JEOL JMS-SX/SX 102A mass spectrometer. The temperature was raised by steps of 128 °C/min from 100 to 300 °C.

Statistical Analysis. Statistical analyses were done according to the SAS (1985) *User's Guide*. Analyses of variance were performed by ANOVA procedure. Significant differences between the means were determined by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Table 1 shows the proximate analysis of mulberry leaves. Among the constituents, the nitrogen-free extract was the highest amount followed by the crude protein fraction. The yields of different organic solvent extracts from 10 g of freeze-dried powder are shown in Table 2. The results indicate that the yield of extract increases with increasing polarity of solvent. Apparently, methanol is the most effective in extraction of antioxidants from mulberry leaves followed by acetone. This is in agreement with the report of Economou et al. (1991) that methanol is a widely used and effective solvent for extraction of antioxidants.

Table 2. Yield of Extracts from Mulberry Leaves with Various Solvents

solvent	yield,[a] g	solvent	yield
methanol	2.26 ± 0.15A	ethyl acetate	0.80 ± 0.03C
acetone	1.78 ± 0.10B	*n*-hexane	0.48 ± 0.02D

[a] Based on 10.0 g of freeze-dried mulberry leaves for each organic solvent. Values are means ± standard deviation of three replicate analyses. Means within a column with different letters are significantly different at $P < 0.05$.

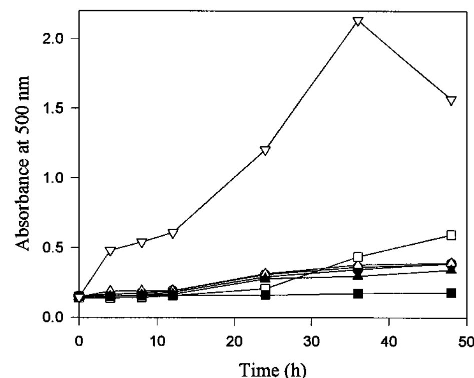

Figure 1. Antioxidant activity of mulberry extracts from various solvents. Concentration of each sample was 200 ppm. (○) Acetone extracts; (●) *n*-hexane extracts; (△) methanol extracts; (▲) ethyl acetate extracts; (□) α-tocopherol; (■) BHA; (▽) blank.

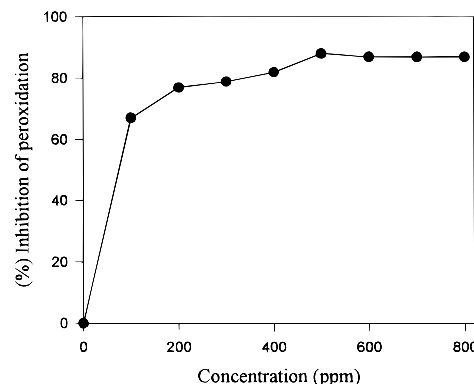

Figure 2. Antioxidant activity of methanolic extracts from mulberry leaves with different concentrations.

Comparison of antioxidant activity of mulberry leaves extracts in various solvents with commercial antioxidants BHA and α-tocopherol is shown in Figure 1. Mulberry leaf extracts with various solvents exhibited strong antioxidant activity. Furthermore, no significant difference ($P > 0.05$) was found in antioxidant activity among the four extracts. This result indicated that mulberry leaf extracts with various solvents displayed a similarly strong antioxidant activity. The antioxidant activity of the extracts was slightly less than that of BHA but was stronger than that of α-tocopherol. Among the four organic solvents, MEML exhibited the highest yield. Therefore, we used this extract as the antioxidant in the following study. As shown in Figure 2, the antioxidant activity of MEML increased with

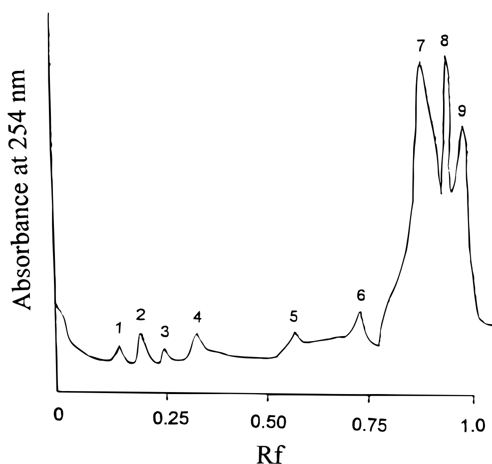

Figure 3. TLC profile of methanolic extracts from mulberry leaves as observed by TLC scanner (254 nm). Solvent system: benzene/ethyl formate/formic acid (75:24:1 v/v/v).

Table 3. Some Characteristics of Different Fractions of Methanolic Extracts from Mulberry Leaves Separated by Silica Gel TLC

fraction	R_f[a]	UV 254 nm		vis		FPT[c]
		color	strength[b]	color	strength[b]	
I	0.92	yellow	+++++	yellow	+++++	78
II	0.86	yellow	++	yellow	++	71
III	0.80	red	+++	green	+++	60
IV	0.75	red	+++	green	+++	52
V	0.53	red	+			21
VI	0.29	red	++			33
VII	0.27	yellow	++			30
VIII	0.20	red	+			25
IX	0.17	red	+			28

[a] Solvent system: benzene/ethyl formate/formic acid (75:24:1 v/v/v). [b] Strength is the depth of color: +++++, very heavy; ++++, heavy; +++, intermediate; ++, slight; +, very slight. [c] FPT is the fluorescence persisting time (h) under UV irradiation.

increasing concentration up to 500 ppm, and then no significant differences ($P > 0.05$) were found in antioxidant activity with concentration from 500 to 800 ppm. This result indicates that 500 ppm of MEML exhibited strong antioxidant activity (88.0%) equal to that of antioxidant activity of 200 ppm BHA (88.4%).

With the BEF solvent system, MEML separated into nine UV-distinct fractions (Figure 3). The characteristics of all fractions are shown in Table 3. The four fractions with $R_f = 0.92$ (I), 0.86 (II), 0.80 (III), and 0.75 (IV) produced intense coloration, and the yields of extraction were 14.2, 12.6, 11.5, and 7.9%, respectively. The FPTs of $R_f = 0.92$, 0.86, 0.80, and 0.75 were 78, 71, 60, and 52 h, respectively, indicating that fractions I–IV exhibited stronger antioxidant activity than the others. In addition, antioxidant activity was found in fractions I, II, III, and IV at 77.3, 72.2, 50.7, and 2.7% inhibition on peroxidation of linoleic acid, respectively (Figure 4). This was similar to the result of FPT as shown in Table 3. Furthermore, 200 ppm of fractions I and II exhibited stronger antioxidant activity than 200 ppm of α-tocopherol. The active fractions I and II were further purified on a silica gel plate by using a toluene/ethyl formate/formic acid (66:29:5 v/v/v) (TEF) solvent system. The characteristics of different subfractions of fraction I separated with TEF solvent system by TLC are shown in Table 4. Four subfractions were observed and designated Ia ($R_f = 0.87$), Ib ($R_f = 0.77$), Ic, ($R_f = 0.73$), and Id ($R_f = 0.65$). Among the four subfractions, Ia and Ib had 74.2 and 18.9% yield and 54 and 28 h FPTs,

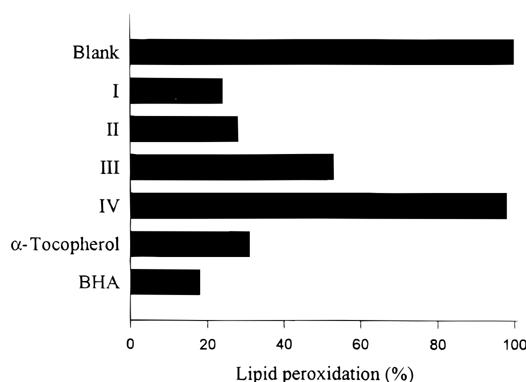

Figure 4. Antioxidant activity of different fractions of methanolic extracts from mulberry leaves separated by silica gel TLC. Concentration of each sample was 200 ppm.

Table 4. Some Characteristics of Different Subfractions of Fraction I of Methanolic Extracts from Mulberry Leaves Separated by Silica Gel TLC

fraction	R_f[a]	UV 254 nm		vis		FPT[c]
		color	strength[b]	color	strength[b]	
Ia	0.87	yellow	+++++	yellow	+++++	54
Ib	0.77	yellow	++	yellow	++	28
Ic	0.73	red	++	green	++	17
Id	0.65	red	++	green	++	9

[a] Solvent system: toluene/ethyl formate/formic acid (66:29:5 v/v/v). [b] Strength is the depth of color: +++++, very heavy; ++++, heavy; +++, intermediate; ++, slight; +, very slight. [c] FPT is the fluorescence persisting time (h) under UV irradiation.

respectively, indicating Ia and Ib exert a more effective antioxidant activity than the others. In addition, subfractions Ia and Ib exhibited 71.2 and 37.8% inhibition on peroxidation of linoleic acid, respectively.

Accordingly, the existence of markedly strong antioxidant activity and high yield were observed in subfraction Ia. Therefore, the present investigation of purification and identification was focused more on this subfraction. Subfraction Ia was developed on a silica gel plate by using various solvent systems, including TEF, TCA, BG, BA, BGW, and BBF, but no separation was obtained. Additionally, fraction II was also not fractionated into any subfractions by using TEF, TCA, BG, BA, BGW, and BBF. Spots Ia and II yielded powder-yellow and pale yellow viscous materials, respectively.

λ_{max} and ϵ values for fraction Ia in MeOH solution were 453 nm and 1.91×10^5, respectively. The ^{1}H-NMR spectrum of Ia exhibited 28 hydrogens, with the following chemical shifts: δ 1.03 (s, 6H, $-CH_3 \times 2$), 1.40–1.65 (m, 4H, $-CH_2 \times 2$), 1.72 (s, 3H, $-CH_3$), 1.97 (s, 6H, $-CH_3 \times 2$), 2.03 (m, 2H, $-CH_2$), 6.10–6.40 (m, 5H, $-CH=$), 6.60–6.70 (m, 2H, $-CH=$). The ^{13}C-NMR spectrum revealed 20 peaks, with the following chemical shifts: δ 137.95, 137.82, 137.28, 136.52, 136.06, 132.47, 130.88, 130.02, 129.42, 126.68, 125.06, 39.59, 34.22, 33.06, 28.92 (×2), 21.72, 19.20, 12.76, 12.70. The data of mass spectra (m/z) were 536 (M^+, 100), 69 (33.17), 119 (19.20), 157 (10.87), and 444 (18.35). Additionally, the result of the UV–vis absorption spectral analysis of fraction Ia agreed with that of β-carotene described by Silverstein et al. (1991). Furthermore, β-carotene is a symmetrical structure; hence, on the basis of these data, a molecular formula of $C_{40}H_{56}$ was suggested. This was identified as β-carotene.

As for fraction II, λ_{max} and ϵ values in MeOH solution were 296 nm and 1.25×10^6, respectively. The ^{1}H-NMR

spectrum of fraction II exhibited 50 hydrogens, and its chemical shift appeared at δ 4.20 (m, 1H, −OH), 2.60 (t, 2H, $-CH_2-$), 2.17 (s, 3H, CH_3-), 2.12 (s, 6H, $CH_3 \times 2$), 1.80 (m, 2H, $-CH_2-$), 0.97−1.62 (m, 24H, $-CH_2-$), 0.92−0.95 (d, 12H, CH_3-). The ^{13}C-NMR spectrum of fraction II revealed 29 carbons, with the following chemical shifts: δ 11.24, 11.75, 12.20, 19.50, 19.68, 22.57, 22.67, 23.86, 20.64, 21.00, 24.68, 24.39, 31.42, 37.33 (×4), 39.32, 39.78, 27.98, 32.61, 32.67, 74.30, 118.60, 119.21, 120.25, 120.50, 144.20, 144.96. The data of mass spectra (*m*/*z*) were 430 (M^+, 100), 57 (6.25), 165 (63.49), and 205 (9.75). On the basis of these data, a molecular formula of $C_{29}H_{50}O_2$ was proposed, and this was identified as α-tocopherol. The purified fractions Ia and II also were compared with authentic β-carotene and α-tocopherol by HPLC analysis, and the retention times of fractions Ia and II were the same as those of β-carotene and α-tocopherol, respectively (data not shown), indicating that the major antioxidant components of MEML were β-carotene and α-tocopherol.

Tocopherol is a natural antioxidant and β-carotene is also a well-known quencher of singlet oxygen that initiates lipid peroxidation (Foote and Denny, 1968). As mentioned above, β-carotene and α-tocopherol were identified as the major components from mulberry leaves. Apparently, the presence of β-carotene and α-tocopherol in mulberry leaves seems to be related to their antioxidant activity on prevention of lipid peroxidation. Further research on the mechanism and application of the antioxidant effects of MEML is in progress.

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