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Antioxidative Effect and Active Components from Leaves of Lotus (*Nelumbo nucifera*)

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The DPPH scavenging effect, the inhibition of human low-density lipoprotein oxidation, and antioxidative contents were employed for the activity-guided purification to identify the antioxidant components of lotus leaves (leaves of *Nelumbo nucifera* Gaertn.). The methanolic extract of lotus leaves (LLM) was separated into ethyl acetate (LLME), *n*-butanol (LLMB), and water (LLMW) fractions. LLME and LLMB exhibited greater capacity to scavenge DPPH radical, delayed LDL oxidation, and had higher antioxidative contents than LLMW. Seven flavonoids were isolated from both fractions by column chromatography. On the basis of 1D- and 2D-NMR experiments and MS data analyses, these compounds were identified as catechin (**1**), quercetin (**2**), quercetin-3-*O*-glucopyranoside (**3**), quercetin-3-*O*-glucuronide (**4**), quercetin-3-*O*-galactopyranoside (**5**), kaempferol-3-*O*-glucopyranoside (**6**), and myricetin-3-*O*-glucopyranoside (**7**). Quercetin and its glycosides (compounds **2**−**5**) exerted potent inhibition of LDL oxidation, whereas myricetin-3-*O*-glucopyranoside (**7**) showed stronger DPPH scavenging activity. These results indicate that the antioxidant capacity of lotus leaves is partially relevant to its flavonoids.

KEYWORDS: Lotus leaves; *Nelumbo nucifera*; LDL; DPPH; flavonoids

INTRODUCTION

Excessive production of free radicals and reactive oxygen species (ROS) is believed in cellular and tissue pathogenesis to lead to several chronic diseases such as cancer, atherosclerosis, and cardiovascular disease (CVD). Lipid peroxidation of low-density lipoprotein (LDL), which is initiated by ROS, plays a causative role in the early pathogenesis of atherosclerosis (*1*). Moreover, the oxidized LDL (ox-LDL) taken up via the scavenger receptor of macrophages, causing the formation of foam cells, is involved in more advanced stages of atherosclerosis lesions (*2*). Thus, prevention of LDL oxidation and ROS formation possibly attenuates the development of the early stages of atherosclerosis.

Consumption of specific foods and nutrients that contain flavonoids, tocopherol, and ascorbic acid appears to terminate the free radical chain reaction and have beneficial effects on LDL particle oxidation; thus, supplementation of antioxidants or dietary flavonoids may provide a therapeutic strategy to prevent CVD (*3*). The flavonoids are a group of phenolic compounds that are extensively distributed in the plant kingdom such as fruits, vegetables, flowers, and leaves. The biological activities of flavonoids, including anti-inflammatory and antioxidant activities, have been widely investigated (*4*).

Nelumbo nucifera Gaertn. belonging to the Nymphaeaceae family is an aquatic perennial widely planted in eastern Asia and cultivated for food and drink. According to folk medicine, it was traditionally used for dispersing summer heat. Numerous papers have mentioned the pharmacologically and physiologically activities of lotus leaves, including antioxidant (*5*, *6*), anti-HIV (*7*), and antiobesity effects (*8*, *9*). The antioxidant activity of lotus leaves has been reported, but the specific compounds responsible for the antioxidant activity remain unknown (*6*). In this study, first we used successive solvent partitioning to investigate the antioxidant properties and antioxidative contents of different polarity fractions. Second, activity-guided purification was employed to identify and elucidate the active antioxidative constituents in the methanolic extract of lotus leaves. Finally, comparison of their antioxidant capacities was estimated.

MATERIALS AND METHODS

Chemicals and Instruments. All solvents used for extraction and chromatographic isolation were of analytical grade. TLC plates were obtained from Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH), gallic acid, (+)-catechin, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma Chemical (St. Louis, MO). Copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) for LDL oxidation was obtained from Nacalai Tesque

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(Kyoto, Japan). Folin−Ciocalteu's phenol reagent was purchased from Merck. All other reagents were of analytical grade.

NMR spectra were run on a Bruker AVANCE 400 spectrometer. Mass spectra (EIMS) were recorded on a JEOL JMS-100 instrument. Fast atom bombardment mass spectra (FABMS) were taken on a JEOL SX-102A mass spectrometer. Samples were premixed with *m*-nitrobenzyl alcohol (NBA) as the matrix. Xe gas was used as the collision gas of 6−8 kV energy.

Plant Materials. The leaves of *N. nucifera* were purchased from a local farmer in Tainan, Taiwan, in September 2005. After harvest, the leaves were dried at ambient temperature, blended into powder form, and then screened through a 20-mesh sieve (aperture = 0.94 mm). The dried powders were stored at 4 °C before use.

Extraction and Isolation of Effective Compounds from Lotus Leaves. **Figure 1** shows the scheme for the preparation and purification of effective compounds from lotus leaves. In brief, the dried powders of lotus leaves (20 kg) were extracted three times with 50 L of methanol at 100 °C for 1 h. The extract was combined and concentrated to dryness under reduced pressure. The methanolic extract (1.8 kg) was named LLM, suspended in 2.7 L of H_2O, and partitioned successively with the same volume of ethyl acetate and *n*-butanol to yield ethyl acetate-soluble (LLME, 636 g), *n*-butanol-soluble (LLMB, 444 g), and water-soluble (LLMW, 644 g) fractions. The yield of each partition is illustrated in **Table 1**. LLME was subjected to silica gel column chromatography (CC) with a hexane/ethyl acetate (0−100%)/methanol gradient to afford LLME-1−8. The yield of each fraction is shown in **Table 2**. The active fraction LLME-8 (70% EA/Hex−100% MeOH elute) was subjected to Sephadex LH-20 CC with methanol to yield compounds **1** and **2**. The LLMB fraction was also first separated by Sephadex LH-20 CC with methanol elution to obtain fractions LLMB-1−6. The combination was carried out according to the similarity of each collection in TLC. Active fractions LLMB-3−6 were further purified on a Sephadex LH-20 column to yield compounds **2**−**7**. Their structures are shown in **Figure 2**. Each compound was identified by 1D- and 2D-NMR and MS and checked for purity in comparison with authentic samples with purities of 90−95% by TLC and HPLC.

Measurement of DPPH Radical Decoloration Assay. Antioxidant activity was evaluated by a DPPH radical scavenging assay modified from that of Kuo et al. (*10*). A 100 μM DPPH radical solution was prepared in methanol. The reaction was performed by adding 250 μL of DPPH solution with 30 μL of various fractions (final concentrations were 50, 25, 12.5, 6.25, and 3.125 μg/mL) and pure compounds (final concentrations were 1.25 μM), and the mixtures were then kept in the dark for 90 min. The absorbance was measured on a spectrophotometer (Molecular Devices, San Francisco, CA) at 517 nm against a blank of methanol without DPPH. The degree of decoloration indicated the free radical scavenging ability of samples. The EC_{50} (the efficient concentration of extracts and compounds decreasing initial DPPH concentration by 50%) was obtained by interpolation from linear regression analysis. Values are presented as mean ± standard deviation (SD) of three replicates.

Inhibition of Copper Ion-Mediated LDL Oxidation. The method was carried out as previously described by Hsieh et al. (*11*). Blood samples were collected from healthy, nonsmoking male volunteers who had fasted overnight; the samples were then isolated by sequential density ultracentrifugation (d = 1.024−1.050) at 4 °C in a Beckman model LE-80K ultracentrifuge using a 70 Ti rotor (Palo Alto, CA). Isolated LDL was dialyzed overnight at 4 °C with 10 mM sodium phosphate buffer (pH 7.0) to remove the excess of buffer salt. LDL was diluted with PBS to adjust to a concentration of 100 μg of proteins/mL measured by using the Bradford method according to the recommended procedure (Bio-Rad, Canada). The diluted LDL (100 μL) was incubated with 10 μL samples. Oxidation was initiated by the addition of 10 μL of freshly prepared $CuSO_4$ solution (the final concentration was 10 μM), and the final volume of the preparation was adjusted to 250 μL by adding PBS. The kinetics of LDL oxidation was obtained by monitoring the absorbance of conjugated diene formation at 232 nm with a multidetection microplate reader (Synergy HT, BIO-TEC, Atlanta, GA) at 15 min intervals at 30 °C. The lag phase (min) was determined as the intercept of the linear least-squares slope of oxidation curve with the initial absorbance axis.

Determination of Total Phenolic Contents. The total phenolic contents were determined by using the Folin−Ciocalteu assay (*11*). One hundred microliters of a 2000 μg/mL sample was mixed with 2 mL of deionized water and 1 mL of Folin−Ciocalteu phenol reagent. Five milliliters of 20% aqueous sodium carbonate solution (w/v) was added to the mixture, followed by incubation at ambient temperature in the dark for 20 min. The absorbance was measured on a spectrophotometer (Molecular Devices, San Francisco, CA) at 735 nm. The total phenolic contents were determined using gallic acid as a calibration standard at various concentrations (0−800 μg/mL). The results were expressed as millgrams of gallic acid equivalents per gram of sample.

Determination of Total Flavonoid Contents. The total flavonoid contents were determined according to the colorimetric method (*11*). Deionized water (1.25 mL) and 75 μL of 5% $NaNO_2$ were added to 200 μL of a 2000 μg/mL sample. After 6 min, 150 μL of 10% $AlCl_3 \cdot H_2O$ solution was added. After 5 min, 0.5 mL of 1 M NaOH solution was added, and then the total volume was made up to 2.5 mL with deionized water. After the solution was mixed well, the absorbance was measured on a spectrophotometer (Molecular Devices) at 510 nm. The total flavonoid contents were determined using catechin as a calibration standard at various concentrations (0−800 μg/mL). The results were represented as milligrams of catechin equivalents per gram of sample.

Spectral Identification of Purified Compounds. Compounds **1**−**7** were elucidated by spectroscopic methods, including 1D- and 2D-NMR and MS spectral analyses.

*Catechin (**1**):* EIMS, m/z 290 $[M]^+$; ^{1}H NMR (400 MHz, CD_3OD) δ 6.60−6.70 (3H, m, aromatic H), 5.82 (1H, d, J = 2.4 Hz, H-8), 5.75 (1H, d, J = 2.4 Hz, H-6), 4.46 (1H, d, J = 7.2 Hz, H-2), 3.87 (1H, m, H-3), 2.75 (1H, dd, J = 6.4, 16.0 Hz, H-4), 2.40 (1H, dd, J = 8.0, 16.0 Hz, H-4); ^{13}C NMR (400 MHz, CD_3OD) δ 157.8 (s, C-9), 157.5 (s, C-7), 156.8 (s, C-5), 146.2 (s, C-3′), 146.2 (s, C4′), 132.2 (s, C-1′), 120.0 (d, C-6′), 116.1 (d, C-5′), 115.2 (d, C-2′), 100.8 (s, C-10), 96.3 (d, C-6), 82.8 (d, C-8), 82.8 (d, C-2), 68.8 (d, C-3), 28.4 (t, C-4). The data were in agreement with the reported literature values (*12*).

*Quercetin (**2**):* EIMS, m/z 302 $[M]^+$; ^{1}H NMR (400 MHz, CD_3OD) δ 6.17 (1H, d, J = 2.0 Hz, H-6), 6.37 (1H, d, J = 2.0 Hz, H-8), 6.87 (1H, d, J = 8.8 Hz, H-5′), 7.62 (1H, dd, J = 2.0, 8.8 Hz, H-6′), 7.65 (1H, d, J = 2.0 Hz, H-2′); ^{13}C NMR (400 MHz, CD_3OD) δ 175.3 (s, C-4), 163.6 (s, C-7), 160.5 (s, C-5), 156.2 (s, C-9), 146.8 (s, C-4′), 146.7 (s, C-2), 144.2 (s, C-3′), 135.2 (s, C-3), 122.2 (s, C-1′), 119.7 (d, C-6′), 114.2 (d, C-5′), 114.0 (s, C-2′), 102.5 (s, C-10), 97.2 (d, C-6), 92.4 (d, C-8). The data were in agreement with the reported literature values (*13*).

*Quercetin-3-O-glucopyranoside (**3**):* FABMS, m/z 465 $[M + H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.44 (1H, d, J = 7.6 Hz, H-1″), 6.18 (1H, d, J = 2.0 Hz, H-6), 6.38 (1H, d, J = 2.0 Hz, H-8), 6.83 (1H, d, J = 8.4 Hz, H-5′), 7.53 (1H, d, J = 2.0 Hz, H-2′), 7.65 (1H, dd, J = 2.0, 8.4 Hz, H-6′); ^{13}C NMR (400 MHz, DMSO-d_6) δ 177.8 (s, C-4), 164.2 (s, C-7), 161.7 (s, C-5), 156.8 (s, C-2), 156.6 (s, C-9), 148.9 (s, C-4′), 145.2 (s, C-3′), 133.8 (s, C-3), 122.0 (d, C-6′), 121.6 (s, C-1′), 116.6 (d, C-5′), 115.6 (d, C-2′), 104.6 (s, C-10), 101.3 (d, C-1″), 99.1 (d, C-6), 93.9 (d, C-8), 78.0 (d, C-5″), 76.9 (d, C-3″), 74.5 (d, C-2″), 70.4 (d, C-4″), 61.4(t, C-6″). The data were in agreement with the reported literature values (*14*).

*Quercetin-3-O-glucuronide (**4**):* FABMS, m/z 479 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.30 (1H, d, J = 7.2 Hz, H-1″), 6.10 (1H, d, J = 2.0 Hz, H-6), 6.29 (1H, d, J = 2.0 Hz, H-8), 6.80 (1H, d, J = 8.4 Hz, H-5′), 7.34 (1H, dd, J = 2.0, 8.4 Hz, H-6′), 8.02 (1H, d, J = 2.0 Hz, H-2′); ^{13}C NMR (400 MHz, DMSO-d_6) δ 177.4 (s, C-4), 172.3 (s, C-6″), 165.2 (s, C-7), 160.9 (s, C-5), 156.5 (s, C-2), 156.5 (s, C-9), 148.4 (s, C-4′), 144.8 (s, C-3′), 133.8 (s, C-3), 120.7 (d, C-6′), 120.7 (s, C-1′), 117.9 (d, C-5′), 115.4 (d, C-2′), 103.4 (s, C-10), 102.6 (d, C-1″), 98.9 (d, C-6), 93.8 (d, C-8), 76.6 (d, C-3″), 74.2 (d, C-2″), 74.2 (d, C-5″), 71.8 (d, C-4″). The data were in agreement with the reported literature values (*15*).

*Quercetin-3-O-galactopyranoside (**5**):* FABMS, m/z 465 $[M + H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.30 (1H, d, J = 7.2 Hz, H-1″), 6.21 (1H, d, J = 2.0 Hz, H-6), 6.43 (1H, d, J = 2.0 Hz, H-8), 6.82 (1H, d, J = 8.4 Hz, H-5′), 7.53 (1H, d, J = 2.0 Hz, H-2′), 7.65 (1H, dd, J = 2.0, 8.4 Hz, H-6′); ^{13}C NMR (400 MHz, DMSO-d_6) δ 177.5 (s, C-4), 164.1 (s, C-7), 161.2 (s, C-5), 156.3 (s, C-2), 156.3 (s, C-9), 148.4 (s, C-4′), 144.8 (s, C-3′), 133.4 (s, C-3), 122.0 (d, C-6′), 121.1 (s, C-1′), 115.9 (d, C-5′), 115.2 (d, C-2′), 103.9 (s, C-10), 101.7 (d, C-1″), 98.6 (d, C-6), 93.5 (d, C-8), 75.8 (d, C-5″), 73.1 (d, C-3″), 71.2 (d, C-2″), 67.9 (d, C-4″), 60.1 (t, C-6″). The data were in agreement with the reported literature values (*16*).

*Kaempferol-3-O-glucopyranoside (**6**):* FABMS, m/z 449 $[M + H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.45 (1H, d, J = 7.2 Hz, H-1″), 6.42 (1H, d,

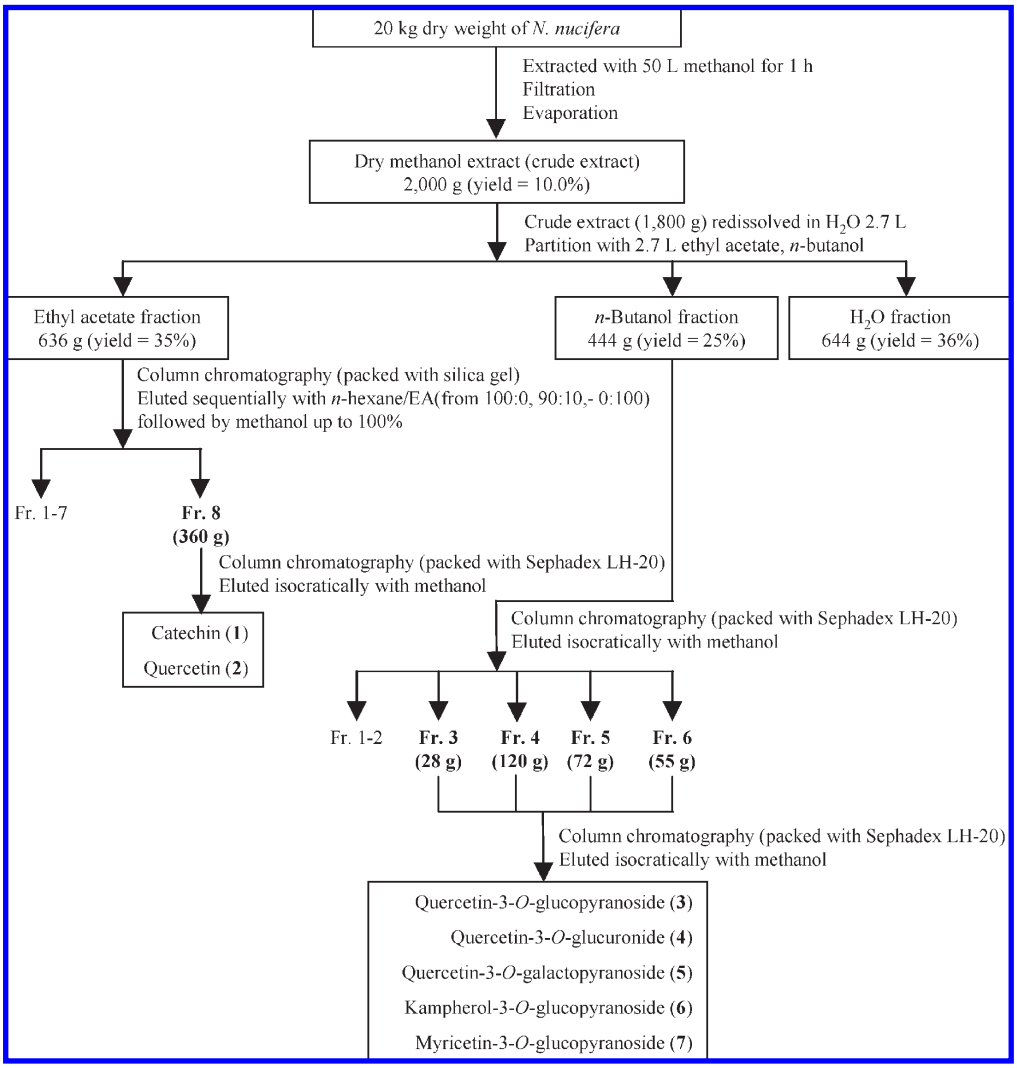

Figure 1. Scheme for the preparation and isolation of antioxidative fractions and compounds from lotus leaves.

Table 1. Antioxidant Activities, Antioxidative Contents, and Yields of Methanolic Extract and Partitions from Lotus Leaves[a]

		Cu^{2+}-induced LDL oxidation				
sample	DPPH scavenging activity EC_{50} (μg/mL)	Δt_{lag}[b] (min)	rel potency of Δt_{lag}[c]	total phenolic content (mg/g)	total flavonoid content (mg/g)	yield (%)
LLM	12.3 ± 0.7b	347 ± 14c	1.18	118.0 ± 7.1b	33.9 ± 1.7a	–[d]
LLME	15.7 ± 1.9c	317 ± 25bc	1.07	128.8 ± 5.9b	67.9 ± 8.0b	35
LLMB	9.7 ± 0.4a	466 ± 20d	1.58	307.8 ± 10.1c	101.8 ± 15.9c	25
LLMW	29.1 ± 0.9d	177 ± 14a	0.60	62.7 ± 1.8a	nd[e]	36
Trolox	–	295 ± 16b	1.00	–	–	–

[a] Values are presented as mean ± SD of three replicates. Values in each column with different letters are significantly different at $p < 0.05$. [b] Δt_{lag} was defined as the difference of time between the samples and copper-ion induced group. The concentration of various samples was 5 μg/mL. [c] The relative potency of Δt_{lag} was represented as Δt_{lag} of samples compared to that of Trolox. The value of 1.00 is expressed in terms of 5 μg/mL of Trolox. [d] –, not determined. [e] nd, not detected.

Table 2. Antioxidant Activities, Antioxidative Contents, and Yields of the Subfractions of LLME from Lotus Leaves [a]

sample	DPPH scavenging activity EC_{50} (μg/mL)	Cu^{2+}-induced LDL oxidation (rel potency of Δt_{lag})[b]	total phenolic content (mg/g)	total flavonoid content (mg/g)	yield (%)
Trolox	–[c]	1.00b	–		–
LLME-1	>50	<0.6	23.2 ± 1.1a	nd[d]	9
LLME-2	>50	<0.6	33.6 ± 2.3ab	nd	6
LLME-3	>50	<0.6	25.1 ± 1.4a	nd	6
LLME-4	>50	<0.6	30.1 ± 0.3ab	nd	9
LLME-5	>50	<0.6	31.6 ± 0.2ab	nd	3
LLME-6	>50	<0.6	41.8 ± 2.6b	nd	2
LLME-7	34.7 ± 3.4	0.66a	200.0 ± 17.0c	159.1 ± 23.3	2
LLME-8	26.6 ± 1.4	1.31c	241.8 ± 15.6d	200.7 ± 33.7	60

[a] Same as **Table 1**. [b] The concentration of various samples was 5 μg/mL. [c] –, not determined. [d] nd, not detected.

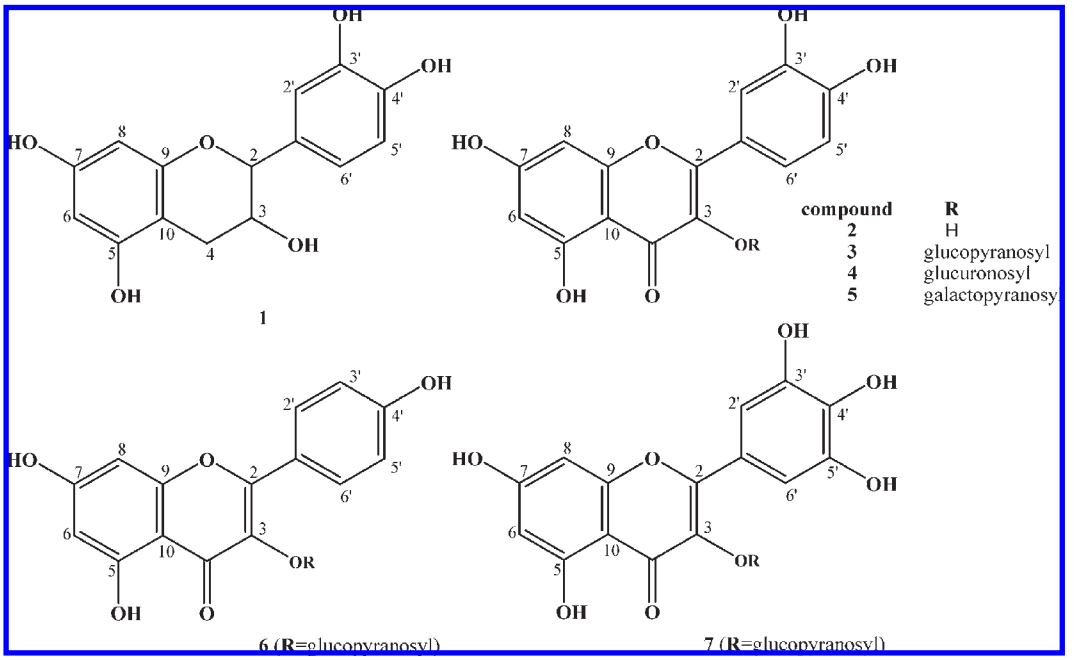

Figure 2. Chemical structures of the antioxidative compounds isolated from lotus leaves.

$J = 2.0$ Hz, H-8), 6.19 (1H, d, $J = 2.0$ Hz, H-6), 6.86 (2H, d, $J = 8.4$ Hz, H-3′,5′), 8.03 (2H, d, $J = 8.4$ Hz, H-2′,6′); ^{13}C NMR (400 MHz, DMSO-d_6) δ 177.4 (s, C-4), 164.1 (s, C-7), 161.1 (s, C-5), 159.9 (s, C-4′), 156.3 (s, C-2), 156.2 (s, C-9), 133.1 (s, C-3), 130.8 (d, C-2′), 130.8 (d, C-6′), 120.8 (s, C-1′), 115.1 (s, C-3′), 115.1 (d, C-5′), 103.9 (s, C-10), 100.8 (d, C-1″), 98.6 (d, C-6), 93.6 (d, C-8), 77.4 (d, C-5″), 76.4 (d, C-3″), 74.1 (d, C-2″), 69.8 (d, C-4″), 60.8 (t, C-6″). The data were in agreement with the reported literature values (*14*).

Myricetin-3-O-glucopyranoside (7): FABMS, m/z 481 $[M + H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.04 (1H, d, $J = 7.2$ Hz, H-1″), 6.18 (1H, d, $J = 2.0$ Hz, H-6), 6.36 (1H, d, $J = 2.0$ Hz, H-8), 7.18 (2H, d, $J = 2.0$ Hz, H-2′,6′); ^{13}C NMR (400 MHz, DMSO-d_6) δ 177.4 (s, C-4), 164.0 (s, C-7), 161.1 (s, C-5), 156.3 (s, C-2), 156.2 (s, C-9), 145.3 (s, C-3′), 145.3 (s, C-5′), 136.4 (s, C-4′), 133.4 (s, C-3), 120.0 (s, C-1′), 108.3 (d, C-2′), 108.3 (d, C-6′), 103.5 (s, C-10), 100.1 (d, C-1″), 98.6 (d, C-6), 93.3 (d, C-8), 77.6 (d, C-5″), 76.6 (d, C-3″), 74.8 (d, C-2″), 69.9 (d, C-4″), 61.1 (t, C-6″). The data were in agreement with the reported literature values (*14*).

Analysis and Quantification of Flavonoids by High-Performance Liquid Chromatography (HPLC) (Figure 3). Analysis was performed with a Hitachi L-6200 intelligent pump equipped with a photodiode array detector (Hitachi L-7455) and a C18 reverse-phase column (150 × 4.6 mm, 5 μm) at room temperature. The mobile phase was composed of two solvents: water containing 2% acetic acid (solvent A) and water containing 0.5% acetic acid/acetonitrile (1:1, v/v, solvent B). Elution conditions were as follows: initial conditions were 5% solvent B; 0−10 min, 5−10% solvent B; 10−40 min, 10−40% solvent B; 40−55 min, 40−55% solvent B; 55−60 min, 55−80% solvent B; 60−65 min, 80−100% solvent B; 65−70 min, 100−50% solvent B; 70−75 min, 50−30% solvent B; 75−80 min, 30−10% solvent B; 80−85 min, 10−5% solvent B; transferred to initial conditions (5% solvent B). The flow rate was 1 mL/min. The quantity of seven flavonoids was calculated from the respective peak area according to individual standard curve.

Statistical Analysis. The results are presented as mean ± SD. Data were analyzed by one-way ANOVA procedures. Duncan's new multiple-range test was used to determine the difference of means, and $p < 0.05$ was considered to be statistically significant.

RESULTS

Antioxidative Ability of LLM and Three Partitions. DPPH has been extensively used as a useful reagent to evaluate radical scavenging ability. The mechanism of scavenging ability was performed by trapping the unpaired electron, and the degree of decoloration indicated the scavenging efficiency of substances. The scavenging activity (EC_{50}) of crude extract and three partitions is demonstrated in **Table 1**. LLMB gave the greatest activity with an EC_{50} value of 9.7 ± 0.4 μg/mL in the DPPH assay, followed by LLM and LLME, whereas LLMW showed the weakest capacity with an EC_{50} value of 29.1 ± 0.9 μg/mL.

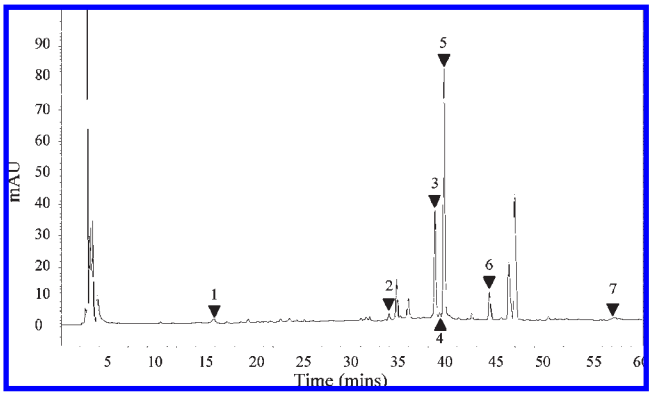

Figure 3. HPLC chromatogram of seven flavonoids from lotus leaves. Peaks: 1, catechin (**1**); 2, myricetin-3-*O*-glucopyranoside (**7**); 3, quercetin-3-*O*-glucopyranoside (**3**); 4, quercetin-3-*O*-galactopyranoside (**5**); 5, quercetin-3-*O*-glucuronide (**4**); 6, kaempferol-3-*O*-glucopyranoside (**6**); 7, quercetin (**2**).

The duration of lag time represented the resistance of lipoprotein to oxidation and is also shown in **Table 1**. LLMB showed the significantly greatest inhibitory effect of delaying LDL oxidation at 1.58 times better than that of Trolox at the same concentration (5 μg/mL). LLME possessed an activity similar to that of LLM; these activities were 1.07 and 1.18 times better than that of Trolox, respectively. Moreover, the inhibitory effects of samples keep a concentration-dependent manner from 5 to 0.625 μg/mL (data not shown).

Antioxidative Contents of LLM and Three Partitions. Foods containing phytochemicals such as polyphenolic compounds or flavonoids have protective potential against degenerative diseases. In an attempt to elucidate the antioxidant activity of lotus leaves, the antioxidative contents were determined. **Table 1** also

Table 3. Antioxidant Activities, Antioxidative Contents, and Yields of the Subfractions of LLMB from Lotus Leaves [a]

sample	DPPH scavenging activity EC_{50} (μg/mL)	Cu^{2+}-induced LDL oxidation (rel potency of Δt_{lag})	total phenolic content (mg/g)	total flavonoid content (mg/g)	yield (%)
Trolox	–	1.00a	–	–	–
LLMB-1	15.3 ± 0.7c	1.05ab	159.1 ± 6.0a	5.4 ± 3.8a	11
LLMB-2	16.0 ± 0.4d	1.12b	182.2 ± 7.7a	9.7 ± 4.4a	20
LLMB-3	4.3 ± 0.1b	1.74c	441.8 ± 15.2b	203.7 ± 54.0bc	7
LLMB-4	3.8 ± 0.4ab	1.87d	523.9 ± 46.0c	180.1 ± 16.7b	30
LLMB-5	3.5 ± 0.3a	1.96d	594.4 ± 37.8d	235.5 ± 29.8c	18
LLMB-6	3.5 ± 0.3a	1.92d	526.8 ± 26.4c	197.0 ± 18.9bc	14

[a] Footnotes *a*–*c* are the same as in **Table 2**.

Table 4. Antioxidant Activities of Seven Flavonoids Isolated from Lotus Leaves and the Contents in LLM [a]

sample	DPPH scavenging activity at 12.5 μM	Cu^{2+}-induced LDL oxidation (rel potency of Δt_{lag})[b]	retention time (min)	content in LLM (mg/g)
Trolox	–	1.00b	–	–
compd **1**	75.6 ± 0.6b	1.86d	16.06	14.5 ± 2.0
compd **2**	91.0 ± 0.6d	2.64f	52.55	4.6 ± 0.4
compd **3**	88.0 ± 2.5cd	2.32e	38.89	42.1 ± 1.8
compd **4**	74.4 ± 2.0b	1.90d	39.83	70.3 ± 2.7
compd **5**	83.8 ± 3.5c	2.25e	39.37	4.2 ± 0.7
compd **6**	15.6 ± 4.9a	0.47a	44.51	8.5 ± 1.4
compd **7**	96.7 ± 2.5e	1.63c	34.16	5.0 ± 1.2

[a] Same as footnote *a* in **Table 1**. [b] The concentration of individual compounds was 1.25 μM. The concentration of Trolox was 5 μg/mL.

illustrates the content of total polyphenols, total flavonoids in LLM, and three solvent partitions. LLMB contained the most abundant amount of both antioxidative contents (307.8 ± 10.1 of gallic acid equiv/g of extract for total polyphenols, 101.8 ± 15.9 of catechin equiv/g of extract for total flavonoids). In addition, the total flavonoids between LLM and LLME showed a significant difference. Moreover, the ratios of total flavonoid/total phenolic content in the LLME and LLMB fractions are about 0.52 and 0.33. Thus, the results suggest that both fractions contained high levels of total flavonoids as a proportion of the total phenolic compounds and indicated that flavonoids may act as abundant and important antioxidants in both fractions. Therefore, LLME and LLMB were selected for further investigation by virtue of their better antioxidant activity and higher antioxidative contents than LLMW. The copper-induced LDL oxidation and DPPH scavenging activity were employed for bioassay-guided fractionation to find the effective subfractions and pure compounds.

Effect of Subfractions from LLME/LLMB on Copper Ion-Mediated LDL Oxidation and DPPH Decoloration Assay. To further investigate the active components in lotus leaves, LLME and LLMB were chromatographed successively on silica gel and Sephadex LH-20 column to obtain eight and six subfractions as shown in **Figure 1**, respectively. The antioxidant activity and antioxidative contents of subfractions are shown in **Tables 2** and **3**. Among the LLME subfractions, the nonpolar subfractions LLME-1–6 showed no activity under the conditions used, only LLME-7 and -8 showed moderate effects against LDL oxidation. In addition, LLME-7 and -8 contained significant higher contents of total phenols and flavonoids than others. For LLMB subfractions, inhibition of LDL oxidation of LLMB-3–6 was 1.74–1.96 times better than that of Trolox and much greater than those of LLMB and LLMB-1 and -2. The antioxidative contents of LLMB-3–6 were significantly higher than others. To explain the inhibition of LDL oxidation, DPPH radical scavenging activity was used to estimate the antioxidant capacity of subfractions. The DPPH scavenging ability of subfractions revealed a parallel trend with LDL oxidation in **Tables 2** and **3**. LLMB-3–6 and LLME-7–8 displayed the better activity, respectively, with EC_{50} values of 3.5–4.3 and 34.7 and 26.6 μg/mL in LLMB and LLME sufractions, respectively, whereas LLME-1–6 showed weak activity (EC_{50} > 50 μg/mL). Furthermore, LLME-8 was selected for further purification but not LLME-7 due to its small amount. LLMB-3–6 were also purified for greater antioxidant activity and higher antioxidative contents.

Isolation of Active Constituents from LLME and LLMB. Bioassay-guided separation of LLME-8 and LLMB-3–6 gave seven flavonoids: catechin (**1**), quercetin (**2**), quercetin-3-*O*-glucopyranoside (**3**), quercetin-3-*O*-glucuronide (**4**), quercetin-3-*O*-galactopyranoside (**5**), kaempferol-3-*O*-glucopyranoside (**6**), and myricetin-3-*O*-glucopyranoside (**7**). These compounds were identified by spectroscopic data (1D- and 2D-NMR and MS) and compared with published data. Their structures are shown in **Figure 2**.

Effect of Pure Compounds on Copper Ion-Mediated LDL Oxidation and DPPH Decoloration Assay. Compounds **1**–**7** were isolated from the effective subfractions and evaluated for their potential to protect human LDL against oxidation in vitro shown in **Table 4**. All compounds displayed the inhibitory effect of LDL oxidation. The seven compounds retarded the LDL oxidation following the order quercetin (**2**) > quercetin-3-*O*- glucopyranoside (**3**) > quercetin-3-*O*-galactopyranoside (**5**) > quercetin-3-*O*-glucuronide (**4**) = catechin (**1**) > myricetin-3-*O*-glucopyranoside (**7**) > kaempferol-3-*O*-glucopyranoside (**6**). Among these compounds, quercetin and its glycosides exhibited more potent antioxidant activity against LDL oxidation at 1.90–2.64 times better than that of Trolox, whereas kaempferol-3-*O*-glucopyranoside had weak activity at 0.47 times. The antioxidant capacity of pure compounds was also evaluated by DPPH assay shown in **Table 4**. The highest activity was found for myricetin-3-*O*-glucopyranoside with 96.7% at 12.5 μM. The order of relative DPPH scavenging effect was similar to that of LDL oxidation besides myricetin-3-*O*-glucopyranoside. These results suggested that the inhibitory ability of antioxidative components in lotus leaf on LDL from oxidation is mostly due to free radical scavenging activity.

Quantitative Analysis of Pure Compounds in LLM by HPLC. The HPLC analysis was conducted to quantitatively estimate the content of seven flavonoids in the LLM fraction at UV 280 nm detection. **Table 4** lists the retention times and contents of seven flavonoids in LLM. The results indicated that

quercetin-3-*O*-glucuronide (**4**) is the most abundant component at 70.3 mg/g of dry weight in LLM, followed by quercetin-3-*O*-glucopyranoside (**3**) at 42.1 mg/g of dry weight in LLM, whereas quercetin (**2**), quercetin-3-*O*-galactopyranoside (**5**), kaempferol-3-*O*-glucopyranoside (**6**), and myricetin-3-*O*-glucopyranoside (**7**) were minor flavonoids at 4.2−8.5 mg/g of dry weight in LLM.

DISCUSSION

There is growing attention to natural antioxidants present in medicinal and dietary plants that might help attenuate oxidative damage and have health-promoting effects in the prevention of degenerative diseases (*17*). The scavenging ability of DPPH free radical is extensively used to screen the antioxidant potential of naturally derived foods. LDL oxidation in the arterial intima can initiate the atherosclerotic process, and α-tocopherol could protect LDL from oxidation (*2*). According to the oxidative modification hypothesis, antioxidants that inhibit the oxidation of LDL are expected to attenuate atherosclerosis (*18*). The measurement of conjugated diene formation is generally applied as a dynamic quantization during the oxidation of LDL (*19*). In the present study both indices were used to evaluate the antioxidant activity of subfractions and isolated pure compounds. The results in previous literature showed that the methanolic extract of the lotus leaves displayed various antioxidant effects on scavenging hydroxyl radicals, metal binding ability, and reducing power as well as against hemoglobin-induced linoleic acid peroxidation (*6*). Methanol was applied in this study to extract the low molecular weight and moderately polar substances because of its wide solubility properties. We found that LLM exhibited the inhibition capacity of LDL oxidation and free radical DPPH scavenging ability (**Table 1**). In this study, we attempted to isolate and characterize the active compounds responsible for antioxidant activities in lotus leaves.

Bioassay-guided fractionation was employed to screen the effective subfractions and pure compounds. The results showed that LLME and LLMB exerted more potential on the retardation of LDL oxidation and free radical scavenging ability as well as antioxidative contents than LLMW (**Table 1**). Several studies have observed that the crude extract and ethyl acetate and *n*-butanol partitions exhibited considerable antioxidant activity. Furthermore, phenolic acids, flavonoids, and their glycosides were widely distributed in such partitions (*10*, *11*, *20*). Moreover, the contents of antioxidant components were correlated with antioxidant activities (*21*). In addition, the flavonoids contributed one-third to half of the total phenolic content (**Table 1**), which indicated that the flavonoids in lotus leaves are important constituents responsible for the bioactivities. However, it seemed that there were some phenolic compounds other than flavonoids involved in lotus leaves. Seven flavonoid compounds were isolated from LLE via bioassay-guided separation in this study. Ohkoshi et al. reported that 3-*O*-α-arabinopyranosyl-(1→2)-*β*-galactopyranoside, rutin, catechin, hyperoside, isoquercitrin, quercetin, and astragalin were isolated from *N. nucifera* and that the flavonoids exhibited lipolytic activity, except rutin and quercetin (*9*). In addition, Kashiwada et al. demonstrated that quercetin 3-*O*-glucuronide revealed less potent anti-HIV activity (*7*). However, in this study we found the seven flavonoids are the dominant active components from *N. nucifera* and exerted inhibitory potency on LDL oxidation. Furthermore, myricetin-3-*O*-glucopyranoside (**7**) was for the first time isolated from *N. nucifera*.

Dietary flavonoids with a catechol structure can reduce LDL peroxidation by scavenging ROS as hydrogen atom donating molecules or as singlet oxygen quenchers, chelation of transition metal ions, and sparing of LDL-associated antioxidants (*22*). Moreover, flavonoids could directly scavenge the free radical and thus protect α-tocopherol from oxidation and reduce the concentration of α-tocopheroxyl radicals (*23*). In this study, lotus leaves are a natural source of flavonoids. Thus, we investigated the effects of these compounds on the susceptibility of LDL to oxidative modification. The seven isolated compounds actually retarded LDL oxidation compared with copper-mediated LDL oxidation (**Table 4**), perhaps through the mechanism described above. In addition, consumption of flavonoid-rich foods may provide synergistic effects of polyphenolic compounds in preventing human LDL oxidation and perhaps benefit cardiovascular diseases (*24*). Therefore, the isolated flavonoid components seem to act interdependently or mutually support activities against LDL oxidation.

To investigate the structure−activity correlation, the seven different flavonoids were assayed for antioxidant properties. For the inhibition of LDL oxidation, the antioxidant potential of flavonoids was based on the chemical structure characteristics, mainly depending on the number and position of hydroxyl groups and the extent of structural conjugations (*25*). Flavonoids bearing an *o*-dihydroxyl functionality had a higher antioxidant potential than those having no such substituent. This is because the oxidation intermediate, *o*-hydroxyl phenoxyl radical, is more stable due to the intramolecular hydrogen bonding interaction and theoretical calculations (*26*, *27*). We verified that compounds **3** and **7** were more efficient than compound **6** in the inhibition of LDL oxidation (**Table 4**), thus supporting those findings. Furthermore, it is clearly seen that the antioxidant capacity of compound **2** is significantly higher than that of compound **1** with the same hydroxyl group arrangement (**Table 4**). This is due to the presence of a carbon 2−3 double bond in conjugation with 4-oxo in the C-ring of flavonoids that is responsible for electron delocalization from the B-ring involved in antioxidant potential (*25*). After hydrogen donation, the phenoxyl radicals are produced and stabilized by the resonance effect of the aromatic nucleus. Moreover, mitochondria may be regarded as important intracellular sources and targets of ROS. The flavonoids bearing the chemical characteristics such as quecertin are major determinants of the antioxidant activity in mitochondria via superoxide scavenging (*28*).

Two pharmacophores present within quercetin contributed to the protection against lipid peroxidation: (1) a catechol moiety in the B-ring and (2) an OH group at the 3-position with electron-donating groups at the 5- and 7-positions in the AC-ring (*29*). Furthermore, quercetin bearing 3′,4′-dihydroxy substitution in the B-ring is shown to be important for Cu^{2+} chelate formation, and a 3-hydroxy group could enhance the oxidation of quercetin (*30*). This emphasizes that the reactivities in enhancing the resistance of LDL oxidation partially depend on interaction with Cu^{2+} ions, whether by chelation or oxidation. On the other hand, we found that quercetin has more antioxidant potency than its glycosides (**Table 4**). The glycosides are appreciably less active than their parent aglycones because of their greater hydrophilicity, which makes them difficult to react with lipid peroxyl radicals inside the LDL particles (*31*). However, quercetin is usually not present in plasma; instead, it is rapidly metabolized during absorption via methylation, glucuronidation, and sulfation. In addition, the conjugated metabolites could be responsible for the in vivo protective activity of quercetin on endothelial dysfunction involved in atherosclerosis (*32*).

According to the DPPH scavenging ability we found that the more hydroxyl substitutions in the flavonoid structure, the stronger the scavenging activity against DPPH radical. This might due to the measurement of the ability of an antioxidant

to donate a hydrogen atom to reduce the DPPH radical. Therefore, it is not a surprise that compound **7**, containing five hydroxyl groups, had higher activity than the others in the DPPH scavenging system (**Table 4**). In contrast, compound **6**, containing three hydroxyl groups, performed less well in the DPPH assay.

In summary, lotus leaves potentially provide benefits for human health because of antioxidant activities. The activity-guided fractionation and identification are employed to isolate seven flavonoids. At least, the antioxidant activities partially contributed to them or synergetic effects. In Taiwan, lotus leaves are usually used for drinking. This study suggested that lotus leaves can provide valuable functional ingredients.

ABBREVIATIONS USED

LLM, methanolic extract of lotus leaves; LLME, ethyl acetate fraction of methanolic extract of lotus leaves; LLMB, *n*-butanol fraction of methanolic extract of lotus leaves; LLMW, water fraction of methanolic extract of lotus leaves; LDL, low-density lipoprotein; DPPH, 2,2′-diphenyl-1-picrylhydrazyl; NMR, nuclear magnetic resonance; MS, mass spectra; ROS, reactive oxygen species; CVD, cardiovascular disease; ox-LDL, oxidized low-density lipoprotein; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; CC, column chromatography; SD, standard error.

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