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Antioxidative Activity and Active Components of Longan (*Dimocarpus longan* Lour.) Flower Extracts

MENG-CHIEH HSIEH,[†] YI-JANE SHEN,[†] YUEH-HSIUNG KUO,[§,#] AND LUCY SUN HWANG*,[†]

Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan 106; Institute for Traditional Medicine, College of Pharmacy, China Medical University, Taichung, Taiwan 404; and Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan 115

Three different solvent extracts (methanol, ethyl acetate, and *n*-hexane) of longan (*Dimocarpus longan* Lour.) flowers were assayed with three different antioxidant capacity methods, namely, the DPPH free radical scavenging effect, the oxygen radical absorbance capacity (ORAC) assay, and the inhibition of Cu^{2+}-induced oxidation of human low-density lipoprotein (LDL). It was revealed that the methanol extract has the best antioxidative activity, followed by ethyl acetate and *n*-hexane extracts. The methanol extract was separated by liquid−liquid partition into *n*-hexane, ethyl acetate, *n*-butanol, and water fractions. The ethyl acetate fraction was found to have the highest activity of delaying LDL oxidation. After silica gel column chromatography, the fraction having a superior activity was identified as containing two major compounds, (−)-epicatechin and proanthocyanidin A2.

KEYWORDS: Longan flower; epicatechin; proanthocyanidin A2; antioxidant; LDL oxidation

INTRODUCTION

Oxidative damage plays an important role in the pathology of human diseases including cancer, diabetes, and cardiovascular diseases (*1*). Numerous dietary natural antioxidants have been reported to protect humans from the attack of reactive oxygen species and delay the progress of certain chronic diseases (*2*).

The most abundant and common dietary antioxidants belong to the family of polyphenolic compounds, in particular, flavonoids, which are widely distributed in the plant kingdom. Among different flavonoids, proanthocyanidins have received quite significant interest due to their observed health benefits (*3*). Proanthocyanidins are molecules composed of monomeric units of flavan-3-ol in dimer, trimer, or polymer forms.

Dimocarpus longan Lour., known as longan (dragon eye) in the Orient, belongs to the Sapindaceae family. Longan is a subtropical fruit widely grown in China and Southeast Asia. The fruit of longan has high commercial value due to its delicate flavor and sweet taste. The honey from longan pollen is the most desirable variety of honey in Taiwan and China. The male flower of longan is an agriculture waste material. It falls to the ground after pollination. In traditional Chinese medicine the flowers are used for the treatment of leucorrhea and kidney disorders (*4*).

The components and their radical scavenging activities of longan seeds, fruit, pulp, and pericarp have been reported (*5*–*8*). Recently, we reported that a fraction of longan flower extract rich in proanthocyanidins has potent antioxidative and anti-inflammatory activities (*9*). However, the structures of active components in longan flowers have not been fully characterized. In the present paper, we report the study of active antioxidative compounds in longan flowers, especially the potential compounds that contribute beneficially to atherosclerosis by inhibition of human low-density lipoprotein (LDL) oxidation.

MATERIALS AND METHODS

Plant Material. The male flowers of *D. longan* Lour. (longan) were supplied by the Farmer's Association (Nanhua Township, Tainan, Taiwan). They were freshly harvested in April 2005 and dried outdoors under direct sunlight until dryness (water content < 15%) to obtain the dried flowers. The dried flowers were stored at 4 °C before use.

Chemicals. All organic solvents used for extraction and column chromatography were of analytical grade. Methanol (anhydrous) for HPLC analysis was of HPLC grade from Mallinckrodt Baker (Phillipsburg, NJ). Methanol-d_4 (99.8 atom % D) was purchased from Sigma Chemical Co. (St. Louis, MO). Aluminum chloride, L-ascorbic acid, (+)-catechin, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), (−)-epicatechin, gallic acid, α-tocopherol, and fluorescein sodium salt were also purchased from Sigma Chemical Co. Folin−Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Aldrich (Milwaukee, WI). (±)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka (St. Gallen, Switzerland).

* Corresponding author (telephone +886-2-2362-9984; fax +886-2-3366-4113; street address No. 1, Roosevelt Rd., Sec. 4, Taipei, Taiwan 10617; e-mail lshwang@ntu.edu.tw).
[†] National Taiwan University.
[§] China Medical University.
[#] Academia Sinica.

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Preparation of Crude Extract. Dried longan flowers were ground in a rotor speed mill (model RT-08, Yuanmei Ltd., Taipei, Taiwan) and filtered through a 40-mesh sieve. Finely ground flower powders were extracted with methanol, ethyl acetate, and *n*-hexane at room temperature for 24 h. The ratio of flower powders to solvent was 1:20 (w/v). The resulting slurries were filtered through a Whatman no. 1 filter paper. This procedure was repeated twice for the residue, and the filtrates were combined. All of the filtrates were collected and concentrated under vacuum at 50 °C and freeze-dried (Freezone 18, Labconco Ltd., Kansas City, MO). The crude dried extract obtained was used directly for chemical analysis and antioxidant tests.

Measurement of DPPH Radical Scavenging Activity. The method was adapted from that of Espin and others (*10*). Each longan flower extract was dissolved in 80% methanol except the *n*-hexane extract, which was dissolved in 10% DMSO in methanol. To a well in a 96-well flat-bottom microplate was added with 30 μL of sample, 20 μL of methanol and 250 μL of 100 μM methanol solution of DPPH. (+)-Catechin and α-tocopherol were used as reference materials. The absorbance of each sample was measured at 520 nm after the reaction mixture was allowed to stand for 90 min at room temperature in the dark. DPPH free radical scavenging activity was calculated according to the equation

$$\text{DPPH scavenging activity (\%)} = [A_c - (A - A_s)]/A_c \times 100 \quad (1)$$

where A_c is the absorbance of the control DPPH solution, A is the absorbance of sample with DPPH solution, and A_s is the absorbance of sample.

Oxygen Radical Absorbance Capacity (ORAC Method). The automated ORAC assay (*11*) was carried out on a Multiple-detection Microplate Reader (Atlanta, GA) with fluorescent filters (excitation wavelength, 485 nm; emission wavelength, 528 nm). Fluorescein was used as a target of free radical attack, with AAPH as a peroxyl radical generator. Trolox was used as the control standard. Final results were calculated on the basis of the difference in the area under the fluorescein decay curve between the blank and each sample. The antioxidative activity of a sample was determined from its ability to protect the fluorescence of the indicator in the presence of peroxyl radicals. Calculations of the final ORAC values were followed by Prior et al. (*11*).

Inhibition of Cu^{2+}-Induced Oxidation of Human LDL. LDL (d = 1.019−1.063 g/mL) was prepared from the plasma of a fasting healthy man by sequential density ultracentrifugation in Beckman Ultracentrifuge model LE-80K (Beckman Instruments Inc., Palo Alto, CA) at 4 °C (*12*) After centrifugation the main lipoproteins (VLDL, LDL, and HDL) were separated from each other. Isolated LDL was dialyzed against 10 mM PBS overnight at 4 °C in the dark, and purged with nitrogen before use. The cholesterol content of the isolated LDL samples were determined with the CHOD-PAP enzymatic test kit (Merck, Darmstadt, Germany) (*13*) and diluted with PBS (5 mM) to give a final cholesterol concentration of 150 μg/mL. The diluted LDL (100 μL) was incubated with 10 μL samples (25 μg/mL in methanol) or positive control (Trolox, 25 μg/mL in methanol) and 130 μL PBS (5 mM) in the presence of 10 μL of 125 μM $CuSO_4$ (the final concentration was 5 μM). The kinetics of LDL oxidation in the initiation, propagation, and termination processes 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.

Determination of Total Phenolic Compounds. The total phenolic compounds of each fraction were determined (*14*) and expressed as milligrams of gallic acid equivalents per gram of extract or fraction. Each fraction was dissolved in methanol as 2000 μg /mL. Two milliliters of deionized water and 1 mL of Folin−Ciocalteu's phenol reagent were added to 200 μg of each sample. Five milliliters of 20% aqueous sodium carbonate solution (w/v) was added and mixed well, and then the mixture was allowed to stand at ambient temperature for 20 min. Absorbance of the developed dark blue-purple color was measured by spectrophotometer at 735 nm. The content of total phenolic compounds in each fraction was determined using a standard curve prepared with gallic acid at varied concentrations (0, 50, 100, 200, 400, 600, and 800 μg /mL).

Determination of Total Flavonoid Compounds. The total flavonoid compounds of each fraction were determined (*15*) and expressed as milligrams of catechin equivalents per gram of extract or fraction. Each fraction was dissolved in methanol as 500 μg/mL. Deionized water (1.25 mL) and 75 μL of 5% (g/mL) $NaNO_2$ were added to 200 μL of each sample. One hundred and fifty microliters of 10% (g/mL) $AlCl_3 \cdot H_2O$ was added 6 min later. After 5 min, 0.5 mL of 1 M NaOH was added, and the total volume was made up to 2.5 mL with deionized water. The solution was mixed well again, and the absorbance was measured against a blank at 510 nm with a multidetection microplate reader (Synergy HT, BIO-TEC). Absorbance of the developed dark blue-purple color was measured by spectrophotometer at 735 nm. The content of total flavonoid compounds in each fraction was determined using a standard curve prepared with catechin at varied concentrations (0, 50, 100, 200, 400, and 800 μg /mL).

Condensed Tannin Determination with Vanillin−HCl. The method was adapted from that of Julkunen-Titto (*14*). One hundred microliters of crude extracts (2000 μg/mL) was taken and put into tubes covered with aluminum foil. Three milliliters of 4% vanillin (w/v) in methanol was added, and the tubes were shaken vigorously with a mixer. After that, 1.5 mL of concentrated HCl was added and the tubes were shaken again. The absorbance was read at 500 nm after being allowed to stand for 20 min at room temperature. The results were plotted after a (+)-catechin standard made in the same manner. The interference background of the crude extract was corrected by preparing the test without vanillin.

Determination of Total Proanthocyanidin Content. A modified acid/butanol assay (*16*) was adopted for quantification of the total proanthocyanidin content of the methanolic plant extracts. Extract (0.25 mL, 1000 μg/mL) was added to 3 mL of a 95% solution of *n*-butanol/HCl (95:5 v/v) in stoppered test tubes, followed by the addition of 0.1 mL of 10% $NH_4Fe(SO_4)_2 \cdot 12H_2O$−HCl solution. The tubes were incubated for 40 min at 95 °C. A red coloration was developed, and absorbance was read at 550 nm. The proanthocyanidin content was expressed as milligrams of cyanidin chloride equivalent per gram of dry weight of plant material.

Determination of Tocopherol Content by HPLC. The tocopherol content of longan flower extracts was determined by HPLC after saponification. A modified assay of Gimeno et al. (*17*) was used. The injection volume was 20 μL. The mobile phase was methanol/water (98:2, v/v), and the elution was performed at a flow rate of 1.5 mL/min. To determine the compounds in the samples, the working standard solutions were analyzed together with the samples, and peak area ratios were used for calculations following the internal standard method. Detection was performed at 292 nm, and each run lasted 15 min.

Determination of Gallic Acid Content. Analysis was performed with a Phenomenex Luna C18 column (4.6 mm × 250 mm i.d., 5 μm particle size (Torrance, CA) at room temperature. All samples were filtered through a 0.45 μm Millipore filter, and 20 μL was injected. A gradient was used consisting of A (0.9% acetic acid) and B (acetonitrile) at a flow rate of 1.0 mL/min. The initial conditions were 92% A and 8% B. After 40 min, the mobile phase was changed linearly to 76% A and 24% B. After that, the mobile phase was changed linearly to 0% A and 100% B. Then 100% B was continued for 10 min, after which the system reverted linearly over 5 min to the initial conditions (92% A and 8% B). Each run lasted for 80 min. The diode array detector was set to 280 nm for the detection of gallic acid.

Separation and Purification of Extract. The freeze-dried methanol extract (1 g) was redissolved in methanol (1.5 mL), and 20 mL of water was added (methanol/water = 1.5:20, v/v). It was then sequentially partitioned with *n*-hexane, ethyl acetate, and *n*-butanol (same solvent ratio as water). Four resulting fractions were evaporated to dryness under vacuum. The ethyl acetate (EA) fraction was then subjected to silica gel column chromatography using a gradient of hexane/ethyl acetate/methanol. Twenty subfractions were obtained, all of which were assayed for the inhibition of LDL oxidation. Fraction 10 (F10), which showed the best activity, was applied to a Sephadex LH-20 (Amersham

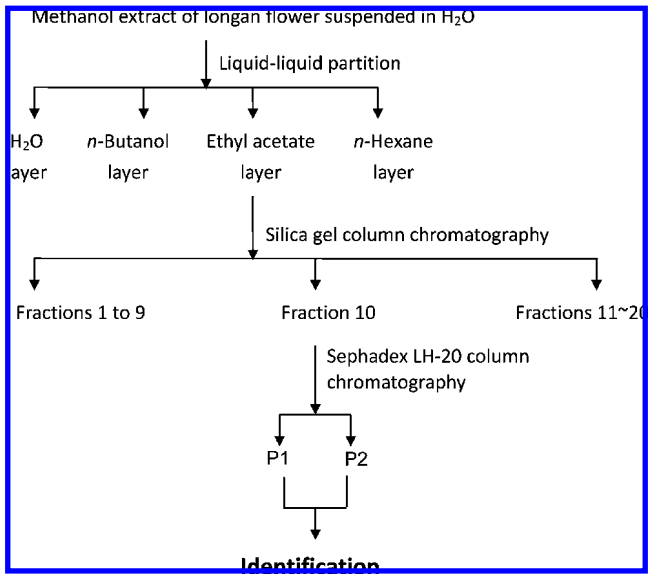

Figure 1. Scheme of the isolation and identification of antioxidative compounds from longan flowers.

Pharmacia Biotech AB, Buckinghamshire, U.K.) column and eluted with methanol to obtain two pure compounds. The scheme of separation and purification is shown in **Figure 1**.

Analysis and Quantification of Compounds by HPLC. The mobile phase was composed of two solvents: A (water) and B (MeOH). Each sample was dissolved in MeOH and filtered through a 0.45 μm Millipore filter. Twenty microliters of each sample was injected. Analysis was performed with a module system using HPLC analytical model 400 pump, model 490 mixer (Applied Biosystems, Foster City, CA) and a Spheris ORB S5ODS2 column (250 mm × 4.6 mm i.d., 5 μm particle size, Waters Corp. Milford, MA) at room temperature using a linear gradient elution. *Elution conditions* were as follows: Solvent B increased from 20 to 80% in 40 min, increased to 100% in 10 min, and then was kept at 100% for 10 min. The flow rate was 1 mL/min. The eluent was monitored at 280 nm by a UV detector model 783-A (Applied Biosystems). The quantity of compounds was calculated from the area under respective peaks according to individual standard curves.

Identification of Purified Compounds. The optical rotations were measured on a JASCO DIP-1000 digital polarimeter (Tokyo, Japan). The IR spectra were recorded on a Nicolet MAGNA-IR 550 spectrometer (Waltham, MA). The UV−visible spectra were obtained by using a Helios Alpha UV−vis spectrophotometer (Thermo Electron Co., Waktham, MA). The ^{1}H NMR and ^{13}C NMR (500 MHz) spectra were recorded using a Bruker Avance-500 MHz FT-NMR spectrometer (Bruker Co., Rheinstetten, Germany). Samples were dissolved in deuterated methanol (CD_3OD). Mass spectra were obtained on a VG Platform ESI/MS system (Micromass Co., Altrincham, U.K.). The ESI interface was used in a negative ion mode, with the following settings: spray voltage, 5 kV; capillary temperature, 350 °C; nitrogen sheath gas pressure, 30 psi, respectively. Experiment was performed by direct infusion at flow rate of 10 μL/min of the sample. The mobile phase was methanol/water containing 0.1% formic acid (50:50, v/v). The flow rate was 0.2 mL/min.

(−)-Epicatechin: $[\alpha]_D^{25} = -50.15°$ (*c* 1.07, MeOH); IR (KBr) ν_{max} 3343, 1626, 1518, 1135 cm^{-1}; UV (MeOH) λ_{max} 208, 281 nm; ^{13}C NMR (CD_3OD, 125 MHz) δ 29.2 (C4), 67.5 (C3), 79.9 (C2), 95.9 (C8), 96.4 (C6), 100.1 (C10), 115.9 (C2′), 115.3 (C5′), 119.4 (C6′), 132.3 (C1′), 145.8, 145.9 (C3′, C4′), 157.4 (C5), 157.6 (C7), 157.9 (C9); ^{1}H NMR (CD_3OD, 500 MHz) δ 2.73 (1H, dd, J = 16.7, 2.6 Hz, H-4eq), 2.85 (1H, dd, J = 16.7, 4.5 Hz, H-4ax), 4.16 (1H, br s, H-3), 4.80 (1H, br s, H-2), 5.91 (1H, d, J = 2.2 Hz, H-6), 5.94 (1H, d, J = 2.2 Hz, H-8), 6.75 (1H, d, J = 8.2 Hz, H-5′), 6.79 (1H, dd, J = 8.2, 1.5 Hz, H-6′), 6.97 (d, J = 1.5 Hz, H-2′); ESI$^-$-MS *m/z* (rel int %) 289 (M − 1, 100), 245 (35), 164 (47), 139 (26) (calcd for $C_{15}H_{14}O_6$).

Proanthocyanidin A2 [epicatechin-(4*β*-8,2*β*-*O*-7)-epicatechin]: $[\alpha]_D^{25} = 47.10°$ (*c* 0.21, MeOH); IR (KBr) ν_{max} 3341, 1622, 1524, 1141; UV (MeOH) λ_{max} 207, 281 nm; ^{13}C NMR (CD_3OD, 125 MHz) δ 29.3 (C4), 29.9 (C4′), 67.0 (C3′), 68.1 (C3), 81.8 (C2′), 96.5 (C6′), 96.6 (C8), 98.3 (C6), 100.2 (C2), 102.4 (C4a′), 104.3 (C4a), 107.2 (C8′), 115.7 (C13), 15.6 (C13′), 116.0 (C10), 116.1 (C10′), 119.8 (C14), 120.4 (C14′), 131.2 (C9′), 132.5 (C9), 145.7(C11), 146.0 (C11′),146.3 (C12′), 146.8 (C12), 152.1 (C8a′), 152.3 (C7′), 154.3 (C8a), 157.0 (C5), 158.1 (C7), 156.6 (C5′); ^{1}H NMR (CD_3OD, 500 MHz) δ 2.75 (1H, dd, J = 17.2, 2.1 Hz, H-4′$_{eq}$), 2.94 (1H, dd, J = 17.2, 4.9 Hz, H-4′$_{ax}$), 4.05 (1H, d, J = 3.4 Hz, H-3), 4.23 (1H, br s, H-3′), 4.40 (1H, d, J = 3.4 Hz, H-4), 4.92 (1H, br s, H-2′), 5.99 (1H,d, J = 2.3 Hz, H-6), 6.06 (1H, d, J = 2.3 Hz, H-8), 6.09 (1H, s, H-6′), 6.80 (1H, d, J = 8.2 Hz, H-13′), 6.81 (1H, d, J = 8.2 Hz, H-13), 6.97 (1H, dd, J = 8.2, 1.9 Hz, H-14′), 7.01 (1H, dd, J = 8.2, 2.1 Hz, H-14), 7.13 (1H, d, J = 2.1 Hz, H-10), 7.15 (1H, d, J = 1.9 Hz, H-10′); ESI$^-$-MS *m/z* (rel int %) 575 (M − 1, 100), 449 (51), 423 (59), 285 (29) (calcd for $C_{30}H_{24}O_{12}$).

Statistical Analysis. Antioxidative activity and antioxidative components in longan flowers were measured in triplicate. The results represented mean ± standard error (SD) of three replicate determinations.

RESULTS AND DISCUSSION

Dried male flowers of longan (*D. longan* Lour.) were used in this study. The crude extracts were prepared by extracting longan flowers with three solvents (methanol, ethyl acetate, and *n*-hexane) at room temperature. The methanol extract had the highest yield (32.42% dry weight of flower), followed by the ethyl acetate extract (2.50%) and the *n*-hexane extract (0.84%). The three different solvent extracts of longan flowers were then tested for the antioxidant activity by various assays.

Antioxidative Activity. Two commonly used antioxidant assays, DPPH and ORAC, were used to evaluate the radical scavenging activity and radical chain breaking capability of longan flower extracts. As shown in **Table 1**, the methanol extract gave the lowest EC_{50} value of 3.81 ± 0.21 μg/mL in the DPPH assay, followed by the ethyl acetate extract. The methanol extract had a similar activity as the positive control (+)-catechin and was better than α-tocopherol. Rangkadilok et al. (*6*) reported that fresh and dried seeds of longan extracts exhibited DPPH radical scavenging activities as good as Japanese green tea extract. Our results demonstrated that flowers of longan also had high DPPH scavenging activity.

Table 1 also shows the data on the assay of ORAC for the longan flower extracts. Again, methanol extract gave the highest ORAC value, followed by the ethyl acetate and *n*-hexane extracts. Both methanol and ethyl acetate extracts showed much higher ORAC values than the positive control, ascorbic acid.

Increasing evidence indicates that oxidatively modified LDL is an important mediator in the pathogenesis of atherosclerosis (*18*). Therefore, considerable attention has been focused on the inhibitory effect of antioxidant on LDL oxidation. It is of great interest to note that the methanol extract of longan flowers also showed the best effect in delaying LDL oxidation as compared to ethyl acetate and *n*-hexane extracts.

Determination of Antioxidative Components. In an attempt to elucidate the antioxidative components in longan flowers, the chemical nature of these components was then determined. **Table 2** lists the contents of total polyphenols, total flavonoids, condensed tannins, gallic acid, and proanthocyanidins in the three solvent extracts of longan flowers. The methanol extract of longan flowers contained the most abundant amount of total polyphenols (509.3 ± 8.9 mg of gallic acid equival/g of dry weight), condensed tannins (111.5 ± 3.1 mg of catechin equiv/g of dry weight), and proanthocyanidins (151.7 ± 2.8 mg of cyanidin chloride equiv/g of dry weight). No detectable amounts of anthocyanins, ascorbic acid, and tocopherols could be found in the extracts of longan flowers.

Table 1. Antioxidative Activities of Longan Flower Extracts[a]

	DPPH free radical scavenging effect, EC_{50} (μg/mL)	ORAC[b] (mM Trolox)	Cu^{2+}-induced LDL oxidation Δt_{lag}[c] (min)
methanol extract	3.81 ± 0.21c	6.19 ± 0.05a	122.0 ± 21.9b
ethyl acetate extract	10.51 ± 0.53a	3.27 ± 0.52b	41.0 ± 9.0c
n-hexane extract	>30	0.44 ± 0.12d	8.7 ± 3.8d
(+)-catechin	3.00 ± 0.21c	−[d]	−
ascorbic acid	−	0.95 ± 0.08c	−
α-tocopherol	8.08 ± 1.10b	−	−
Trolox	−	−	224.3 ± 8.1a

[a] All data are expressed as mean ± SD from three different experiments (each experiment was conducted in triplicate). Data in the same column with different letters are significantly different at $p < 0.05$. [b] Millimolar Trolox solution having the antioxidative capacity equivalent to a 1 mg/mL solution of the sample under investigation. [c] Δt_{lag} was the difference in lag phase (min) between the control and various samples; sample concentration was 1 μg/mL. [d] −, not determined.

Table 2. Contents[a] of Total Polyphenols, Total Flavonoids, Gallic Acid, Condensed Tannins, and Proanthocyanidins in Different Solvent Extracts of Longan Flowers

sample (unit)	methanol extract	ethyl acetate extract	*n*-hexane extract
total polyphenols (mg of gallic acid equiv)	509.3 ± 8.9a	205.5 ± 2.4b	14.8 ± 4.9c
total flavonoids (mg of catechin equiv)	142.5 ± 1.8a	98.9 ± 2.1b	31.1 ± 0.7c
gallic acid (mg of gallic acid)	1.9 ± 0.0a	1.6 ± 0.1a	nd[b]
condensed tannins (mg of gallic acid equiv)	111.5 ± 3.1a	53.6 ± 5.3b	22.4 ± 5.0c
proanthocyanidins (mg of cyanidin chloride equiv)	151.7 ± 2.8a	38.7 ± 2.1b	0.8 ± 0.1c

[a] All data are expressed as mean ± SD on per gram dry weight basis from three different experiments (each experiment was conducted in triplicate). Data in the same row with different letters are significantly different at $p < 0.05$. [b] nd, not detected.

Table 3. Antioxidative Assay and Contents of Total Polyphenols and Total Flavonoids of the Different Liquid−Liquid Partition Fractions of Longan Flower Methanol Extract and Yields of Each Fraction

sample	inhibition of Cu^{2+}-induced oxidation of human LDL (rel potency of Δt_{lag})[a]	total polyphenol[b,c]	total flavonoid[b,c]	yield (%)
Trolox	1.00ab	−	−	−
methanol extract	0.61c	402.1 ± 41.4c	131.9 ± 11.5c	100
n-hexane fraction	0.23d	128.9 ± 4.1d	68.5 ± 2.4d	7.2
ethyl acetate fraction	1.14a	701.7 ± 29.3a	213.7 ± 14.8a	20.2
n-butanol fraction	0.77bc	619.6 ± 24.0b	189.1 ± 21.1b	26.7
water fraction	0.34d	173.0 ± 13.5d	55.6 ± 5.3d	43.8

[a] Relative potency was defined as prolongation of lag phase (Δt_{lag}) as compared to Trolox (1 μg/mL); the concentration of all samples was 1 μg/mL. [b] Same units as in **Table 2**. [c] All data are expressed as mean ± SD from three different experiments (each experiment was conducted in triplicate). Data in the same column with different letters are significantly different at $p < 0.05$.

Because the methanol extract contained 2.5 and 4 times higher amounts of total polyphenols and proanthocyanidins than the ethyl acetate extract, it also showed 2−3 times higher antioxidative activities than the ethyl acetate extract (**Table 1**). The methanol extract of longan flowers was, therefore, selected for further study. The Cu^{2+}-induced oxidation of human LDL assay was used for the activity-guided separation and purification of active compounds in longan flowers.

Fractionation and Purification Guided by Inhibition of Cu^{2+}-Induced Oxidation of Human LDL. The methanol extract of longan flowers was fractionated and purified according to the scheme shown in **Figure 1**. After liquid−liquid partition of longan flower methanol extract with *n*-hexane, ethyl acetate, *n*-butanol, and water, the ethyl acetate fraction showed the highest antioxidative activity (**Table 3**). The effect of delaying LDL oxidation was 1.14 times better than Trolox at the same concentration (1 μg/mL). In addition, the EA fraction had the highest contents of total polyphenols (701.7 ± 29.3 mg of gallic acid/g of dry weight of sample) and flavonoids (213.7 ± 14.8 mg of catechin equiv/g of dry weight of sample).

The ethyl acetate fraction was then subjected to silica gel column chromatography and fractionated with a hexane/ethyl acetate/methanol gradient elution. Twenty subfractions were obtained, all of which were assayed for the inhibition of LDL oxidation and quantified for the total polyphenol and total flavonoid contents. F10 (eluted by ethyl acetate/*n*-hexane = 70:30, v/v) had the highest activity in delaying LDL oxidation (1.72 times better than Trolox at the same concentration level) followed by F11 and F9 as shown in **Table 4**.

F10 was then analyzed by HPLC (**Figure 2**) and shown to consist of two major compounds, P1 and P2, with retention times at 13.7 and 17.5 min, respectively. P1 and P2 were further purified by a Sephadex LH-20 column to yield pure compounds. F9 was also analyzed by HPLC under the same condition as F10, and only one peak appeared on the chromatogram with a retention time of 13.7 min, the same as P1. After further spectroscopic analyses, the major compound of F9 was confirmed to be P1. F11 had less total polyphenols and total flavonoids than F9 and F10. Its HPLC chromatogram showed that it was a complicated mixture. It was not further investigated.

Antioxidative Activities of Purified Compounds P1 and P2. The antioxidative activities of P1 and P2 were examined using the assay of the inhibition of Cu^{2+}-induced oxidation of human LDL. As shown in **Table 5**, both P1 and P2 had superior activities in delaying LDL oxidation, and the lag time of each compound was 1.95 and 2.04 times better than

Table 4. Antioxidative Assays and Contents of Total Polyphenols and Total Flavonoids of the Different Subfractions from M-EA Fraction of Longan Flower Methanol Extract after Silica Gel Column Chromatography

sample	inhibition of Cu^{2+}-induced oxidation of human LDL (rel potency of Δt_{lag})[a]	total polyphenol[b,c]	total flavonoid[b,c]	yield (%)
Trolox	1.00	—	—	—
F1	0.00	nd[d]	nd	0.6
F2	0.00	nd	nd	0.2
F3	0.00	nd	nd	0.2
F4	−0.02	nd	nd	0.3
F5	−0.01	nd	nd	0.3
F6	0.17	92.9 ± 8.4k	35.4 ± 25.2j	0.5
F7	0.36	138.8 ± 1.8j	24.1 ± 19.1j	0.6
F8	1.04	442.3 ± 8.4h	277.9 ± 12.6d	0.6
F9	1.22	970.4 ± 11.2a	732.9 ± 19.0a	8.0
F10	1.72	726.1 ± 6.4bcd	472.2 ± 12.9b	3.0
F11	1.26	714.1 ± 4.7d	362.2 ± 10.6c	5.4
F12	0.74	713.4 ± 8.3d	182.3 ± 5.6ef	25.3
F13	0.70	718.8 ± 17.5cd	178.6 ± 8.2ef	11.4
F14	0.62	689.8 ± 9.8e	190.7 ± 8.7ef	6.7
F15	0.60	618.8 ± 18.2f	151.2 ± 13.9gh	2.8
F16	0.74	741.9 ± 7.2b	169.2 ± 2.1fg	9.4
F17	0.65	619.4 ± 6.9f	172.1 ± 9.7efg	3.6
F18	0.53	431.10 ± 5.48h	132.6 ± 4.8hi	2.1
F19	0.56	536.9 ± 8.8g	119.1 ± 13.3i	12.7
F20	0.24	221.8 ± 3.1i	42.9 ± 5.5j	5.9

[a] Same as in **Table 3**; the concentration of all samples was 1 μg/mL. [b] Same units as in **Table 2**. [c] All data are expressed as mean ± SD from three different experiments (each experiment was conducted in triplicate). Data in the same column with different letters are significantly different at $p < 0.05$. [d] nd, not detected.

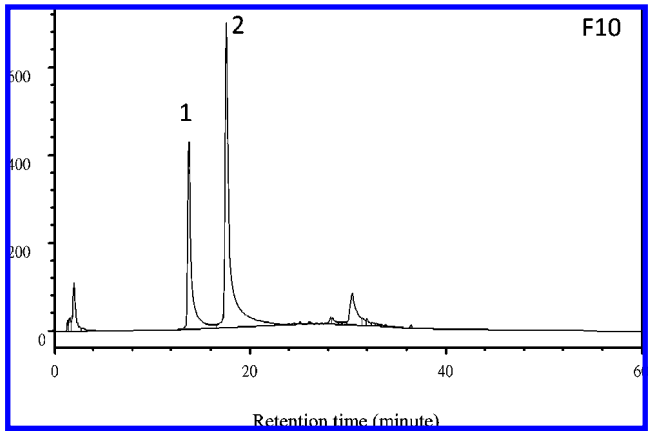

Figure 2. HPLC chromatogram of F10. Peaks: 1, (−)-epicatechin; 2, proanthocyanidin A2.

Table 5. Inhibition of Cu^{2+}-Induced Oxidation of Human LDL by Purified Compounds P1 and P2[a]

sample	Δt_{lag}[b] (min)	rel potency of Δt_{lag}
Trolox	190 ± 36b	1.00
F10	484 ± 93a	2.54
P1	370 ± 71a	1.95
P2	388 ± 114a	2.04

[a] The concentration of all samples was 0.5 μg/mL. [b] Each value represents mean ± SD from three different experiments ($n = 5$ in each experiment). Data in the same column with different letters are significantly different at $p < 0.05$.

that of Trolox at the same concentration level (0.5 μg/mL). However, neither P1 nor P2 was better than F10 (2.54 times better than Trolox).

Identification of P1 and P2. After spectroscopic analyses, P1 was identified as (−)-epicatechin and P2 as proanthocyanidin A2 when their IR, MS, optical rotation, UV−vis, ^{1}H NMR, and ^{13}C NMR data were compared with literature data (*19, 20*). Both (−)-epicatechin and proanthocyanidin A2 were identified in longan flowers for the first time (**Figure 3**). P2, of the rare

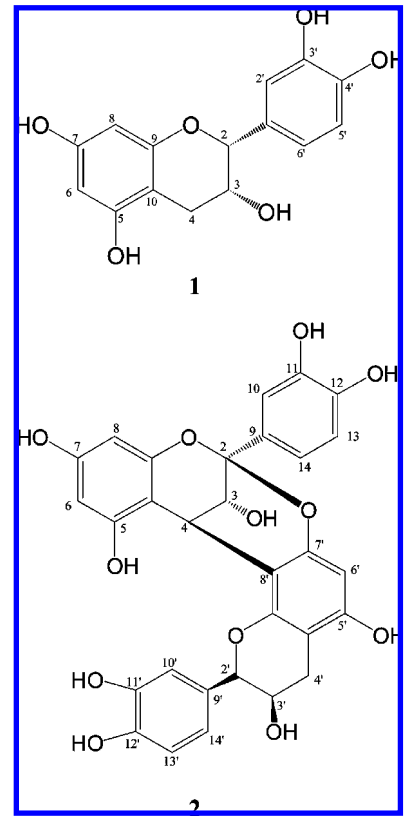

Figure 3. Chemical structures of the antioxidative compounds isolated from longan flowers: (−)-epicatechin (**1**) and proanthocyanidin A2 (**2**).

A-type proanthocyanidins, double linked with their unusual second ether linkage, has been found in some plant foods such as plums, avocados, peanuts, berries, and some uncommon plants (*21*).

No extensive biological activities and active compounds have been reported for longan flowers. Although 13 phenols have been isolated from longan flowers (*22*), the present study reports for the first time on the identification of (−)-epicatechin and

Table 6. Contents of (−)-Epicatechin and Proanthocyanidin A2 in Different Fractions of Longan Flower Extract As Quantified by HPLC

compound	content (% of each fraction)			
	methanol extract	M-EA fraction	F9	F10
(−)-epicatechin	1.39	6.90	78	22
proanthocyanidin A2	0.42	2.10	nd[a]	70

[a] nd, not detected.

proanthocyanidin A2 in longan flowers having superior antioxidant activity.

Catechin and epicatechin are flavonoids that are widely present in many edible plants such as green tea, and numerous studies have dealt with their antioxidative activities in scavenging free radicals, chelating metal ions, preventing the activation of redox-sensitive transcription factors, and inhibiting prooxidant enzymes (*23*).

Previous studies have also demonstrated that proanthocyanidins exhibited biological and pharmacological activities such as anti-inflammatory, antiviral, antibacterial, enzyme-inhibiting, antioxidative, and radical-scavenging properties in vitro (*24*). Proanthocyanidin A2 isolated from cranberry inhibited adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro (*25*).

The number of catechol units was found to positively correlate with the ability of catechins and procyanidins to protect against oxidation of LDL, independently of monomer or chain structure (*26*). Flavonoids can act as potent inhibitors of LDL oxidation via several mechanisms, such as scavenging of free radicals, chelation of transition metal ions, and sparing of vitamin E and carotenoids in the LDL particle (*27*). (−)-Epicatechin has been demonstrated to have ability of inhibiting Cu^{2+} induced LDL oxidation (*28*), and one of the reasons may be involved in the regeneration and protection effects on α-tocopherol in LDL (*29*). The excellent antioxidant activities in inhibition of human LDL oxidation by both (−)-epicatechin and proanthocyanidin A2 from longan flowers may be through the mechanisms described above.

Quantitative Analysis of (−)-Epicatechin and Proanthocyanidin A2 in Longan Flowers. The linear regression equations of standard curves of (−)-epicatechin and proanthocyanidin A2 were established by HPLC at UV 280 nm detection. **Table 6** lists the contents of (−)-epicatechin and proanthocyanidin A2 in different fractions of longan flower extract. It is clear that as the fractionation proceeded, the contents of both compounds increased. F9 contained only (−)-epicatechin, whereas F10 contained more proanthocyanidin A2 than (−)-epicatechin. In the dried longan flowers, the contents of (−)-epicatechin and proanthocyanidin A2 were estimated to be 5.58 and 1.70 mg/g of dry weight from the contents in F9 and F10, respectively.

In conclusion, this study showed that longan flowers contained components with excellent antioxidative activity in the inhibition of human LDL oxidation. The main active components were identified as (−)-epicatechin and proanthocyanidin A2. In Taiwan, dried longan flowers are normally used to make a tea for drinking. Our study provides a useful example of utilizing this byproduct material as a valuable functional ingredient.

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