Isolation, Characterization, and Determination of Antioxidative Activity of Oligomeric Procyanidins from the Seedpod of *Nelumbo nucifera* Gaertn.

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The procyanidins of nonedible parts of lotus (*Nelumbo nucifera* Gaertn.) were determined for the first time. The procyanidins of lotus seedpod were extracted with Me_2CO/H_2O and purified by Sephadex LH-20 column chromatography, with a purity of >98%. ESI-MS analysis showed that the main molecular weight distribution of procyanidins ranged from 291 to 1155, with M + H peak values of 291.1, 579.2, 731.2, 867.2, 1019.4, and 1155.3, respectively. This indicates that the extract contains monomers, dimers, and tetramers of procyanidins, in which the amounts of dimers are greatest, and catechin and epicatechin are the base units. ^1H NMR and ^{13}C NMR spectra confirmed that two to four monomers are linked through C_4-C_8 (or C_6) bonds. The effects of the procyanidins on lipid autoxidation, lipoxygenase activities, and free radical scavenging were also studied. The results showed that 0.1% procyanidins have a strong antioxidant activity in a soybean oil system, better than BHT at the same concentration; inhibited lipoxygenase activity by >90% at a concentration of 62.5 μg/mL, with an IC_{50} value of 21.6 μg/mL; and had IC_{50} inhibitory values rate to •OH of 10.5 mg/L and a scavenging effect on $O_2^{•-}$ of 17.6 mg/L.

KEYWORDS: Lotus seedpod; procyanidins; antioxidant

INTRODUCTION

Procyanidins, also known as condensed tannins, are widely distributed in the plant kingdom and respresent a ubiquitous group of plant phenols. For a long time, the procyanidins of grape seed have been a focus of research around the world. Other plant species have been investigated to explore new sources of procyanidins. The lotus seedpod is the inedible part of *Nelubo nucifera* Gaertn. (*1*). Lotus, as an industrial crop, is widespread in China, and its cultivated area was >40000 ha in 1999. Lotus seed and lotus root are regarded as a popular food and health food, and liensinine, an alkaloid extracted from the lotus seed, is an effective drug to treat arrhythmia. However, lotus seedpod is usually discarded except when sometimes used as a traditional medicine with hemostatic function. Until now, there have been no reports on the isolation and characterization of the lotus seedpod procyanidins extract. In this study, a procyanidin-rich fraction has been isolated from the lotus seedpod of *N. nucifera*, partially characterized by spectroscopic and chemical methods, and tested for its antioxidant activity in vitro.

MATERIALS AND METHODS

Plant materials, including the peduncle, root joints, leaf, and seedpod of *N. nucifera* Gaertn., were collected in Honghu Lantian (Hubei, China) from July of 1999 to September of 1999, and materials at different growth periods of lotus seedpod were collected at the same time. This variety of *N. nucifera* Gaertn. is named Number 2 Wuhan plant and was authenticated by the Department of Botany, Wuhan Plant Institute of the Chinese Academy of Science.

Xanthine, xanthine oxidase, and 2-deoxy-D-ribose were provided by Sigma Chemicals Co. (St. Louis, MO). Procyanidin B2 was purchased from Nakahara Science Co. All other reagents were standard analytical grade items from reputable commercial sources.

Extraction and Isolation. Fresh plant material was lyophilized. To obtain proanthocyanidin polymers representative of those present in intact plant tissues, extreme care was taken to prevent exposure of the preparations to heat, light, and air, and the dry plant material was homogenized three times with acetone/water (7:3), an efficient solvent system for extracting proanthocyanidin polymers (*2*). The combined extracts were concentrated by low-pressure evaporation at <30 °C to eliminate acetone, and the concentrated aqueous solution was extracted again with petroleum ether (5 × 100 mL) and chloroform (5 × 100 mL), respectively, to remove lipids and fat-soluble pigments. The remaining aqueous solution was lyophilized, and one-fourth of the freeze-dried product was dissolved in 10% aqueous methanol. The filtrate was applied to a Sephadex LH-20 column (40 × 2 cm) activated previously with 10% aqueous methanol. The column was eluted with MeOH containing decreasing proportions of H_2O, methanol alone, and finally 50% aqueous acetone to give 100-mL fractions. These fractions were subjected to TLC [silica plates, developed with toluene/Me_2CO/HCO_2H (3:3:1)] (*3*) monitored by $FeCl_3$ and vanillin–hydrochloric acid reagent, and autoxidation activity was determined with *n*-BuOH/HCl/Fe(III) to locate procyanidin-rich fractions, assess their homogeneity, and combine them. These fractions were classified as the total

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procyanidin extract of lotus peduncle (LPPC), joints of lotus root (LRJPC), lotus leaf (LLPC), and lotus seedpod (LSPC).

Spectrometric Quantification. The quantification was transformed into a calibration curve for procyanidin B2 under BuOH/HCl hydrolysis conditions. BuOH/HCl (95:5 v/v) (2.2 mL) and 100 μL of iron reagent (2% w/v solution of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 M HCl) were added to ~5 mg of H_2O/acetone extract in a glass tube and sealed with a Teflon-lined screw cap. After agitation, the tube was heated at 95 °C for 45 min. In addition, the procyanidin content of samples was determined according to the Porter assay (commercial grape seed procyanidins as calibration curve) and the Folin–Ciocalteu assay (commercial tannin as calibration curve) (4−6).

HPLC Analysis. The procyanidins B of lotus seedpod procyanidin extract were analyzed by HPLC as follows: Waters HPLC system, including a model 720 gradient controller, two M510 pumps, a 710B automatic injector, a 440 absorbance detector set at 280 nm, and connected to an Enica21 (Delsi, France) integrator. The column was a 250 × 4.6 mm i.d., 5 μm, reversed-phase Ultrasphere C18 ODS (Beckman, Fullerton, CA) protected with a guard column of the same material and thermostated at 30 °C. The elution conditions were as follows: flow rate, 1 mL/min; solvent A, 2.5% AcOH in H_2O; solvent B, 80% CH_3CN in A; elution starting with 7% B with isocratic elution for 5 min and continuing with a linear gradient from 7 to 20% B in 85 min and from 20 to 100% B in 5 min, followed by washing and reconditioning of the column.
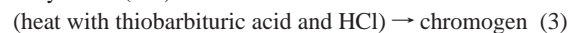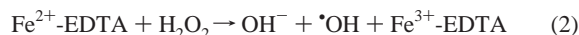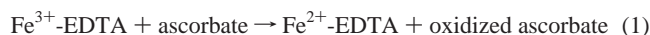
ESI-MS Analysis. The sample (procyanidin extract of lotus seedpod) was analyzed at Beijing Chemical Institute of the Chinese Academy of Sciences. Negative-ion ESI-MS spectra were recorded on an Apex II FT-ICR-MS (Bruker) simple quadrupole mass spectrometer with a nominal mass range up to m/z 2400, equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated with a −4000 V voltage applied to the electrospray needle and a −60 V voltage applied to the orifice. The mass spectrometer was scanned from m/z 200 to 2400, in steps of 0.1 ms unit and with a dwell time of 25 ms at each step. The instrumentation was calibrated using a solution of polypropylene glycol oligomers. Molecular masses are reported as isotopically average values. The procyanidin extract was dissolved in MeOH/H_2O (50:50) at the concentration of 2 mg/mL, and the solution was introduced into the ion spray source at a constant flow rate of 0.2 mL/min with a medical model 22 syringe infusion pump (Harvard Apparatus, South Natick, MA) in combination with a microliter syringe (100 μL).

^{1}H NMR and ^{13}CNMR. For the characterization of the proanthocyanidin polymers, samples (procyanidin extract of lotus seedpod) were dissolved in acetone-d_6/D_2O and analyzed by ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopy using a Bruker DRX500 instrument in Kunming Botany Institute of the Chinese Academy of Sciences.

Deoxyribose Assay To Assess Antioxidant Action. There were two groups in the test, the control group and the sample group. Vitamin C, as positive control, is an effective water-soluble antioxidant. In both groups, different concentrations were tested to find IC_{50} values (concentration at which 50% of $^{\bullet}$OH activity is inhibited) of both vitamin C and procyanidin extract of lotus seedpod.

The deoxyribose method for determining the rate of reaction of hydroxyl radical with a putative antioxidant was performed as described by Halliwell (7). All solutions were prepared in metal-free water. Reactions were performed in 20 mM phosphate buffer, pH 7.4, containing 60 mM deoxyribose, 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2, and the test material. After the addition of ascorbic acid to a final concentration of 100 μM, the reactions started, and the final volume was 1.0 mL. After incubation at 37 °C for 1 h, the color was developed by adding 1.0 mL of 1% thiobarbituric acid (w/v) and 1 mL of 25% HCl, which was then heated in a water bath at 100 °C for 15 min. Samples were allowed to cool. Absorbances were measured at 532 nm and compared to control samples not containing test material and antioxidants. All analyses were run in triplicate and averaged.

In the deoxyribose assay (7), a mixture of Fe^{3+}-EDTA, hydrogen peroxide (H_2O_2), and ascorbic acid at pH 7.4 generates hydroxyl radicals ($^{\bullet}$OH) (eqs 1 and 2), which can be detected by their ability to degrade the sugar deoxyribose into fragments that, on heating with thiobarbituric acid at low pH, generate a pink chromogen.

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{oxidized ascorbate} \quad (1)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}\text{-EDTA} \quad (2)$$

$${}^{\bullet}OH + \text{deoxyribose (DR)} \rightarrow$$
$$(\text{heat with thiobarbituric acid and HCl}) \rightarrow \text{chromogen} \quad (3)$$

Assay of the Scavenging Capacity of Superoxide Radial. Different concentrations of both vitamin C and procyanidin extract of lotus seedpod were tested to find IC_{50} values (content at which 50% of $O_2^{\bullet-}$ activity is inhibited).

Generation of $O_2^{\bullet-}$ by the hypoxanthine−xanthine oxidase system was carried out essentially as described (8, 9), with modifications. Reaction mixtures contained, in a final volume of 3.0 mL made up with 50 mM KH_2PO_4/KOH buffer pH 7.4, cytochrome c (0.1 mM), EDTA (30 μM), hypoxanthine in 0.1 mM KOH (0.4 mM), the compound under test, and xanthine oxidase (0.16 μg/mL freshly diluted). Changes in absorbance were recorded at 550 nm. Due to interference of procyanidins with the assay system, the ability of the compounds to mediate the reduction of cytochrome c directly was compared with that of $O_2^{\bullet-}$ produced by the hypoxanthine−xanthine oxidase system.

Lipid Peroxidation. Pure lard (lard oil, soybean oil) without any additives was used as the substrate to evaluate the antioxidant activity of lotus seedpod procyanidin extract. The procyanidin extract was dissolved in 1.0 mL of 95% ethanol and added to oils at different concentrations (0.01, 0.05, 0.1, and 0.2%), and its antioxidant activity was compared with that of butylated hydroxytoluene (BHT) (0.01 and 0.05%). The control group contained only 1.0 mL of 95% ethanol without any antioxidant. Oil systems were thoroughly homogenized and stored at 60 °C (avoiding light) for 2 weeks. The peroxide value was measured according to AOAC methods (10).

Lipoxygenase Assay. Lipoxygenase activity was analyzed according to the method of Block et al. (11). Linoleic acid (reagent grade) was used as the substrate in a 0.1 M, pH 8.5, Tris buffer at 25 °C. The absorbency at 234 nm was recorded as a function of time on the UV spectrophotometer. The blank control contained all of the reagents except the enzyme solution. Various concentrations of lotus seedpod procyanidin extract were added to the enzyme, and the mixture was incubated at 25 °C for 4 min. The residual enzyme activity and inhibitory rate were then measured as follows:

lipoxyenase activity (units/min/g) =
$$(A_0 \times 200 \times 10)/0.2 \times 4 = 250.0 A_0$$

$$\text{inhibitory rate (\%)} = (1 - A/A_0) \times 100$$

(A_0 = control absorbent value, A = sample absorbent value).

RESULTS AND DISCUSSION

Materials Selection. The procyanidin contents of both different parts of *N. nucifera* Gaertn. and different growing period of lotus seedpod were obtained (**Figure 1**), and they showed that maximum amounts of procyanidins are in the mature lotus seedpod. We extracted procyanidins from lotus seedpod and regarded it as the experimental material resource obtained most easily.

Chemical Character of Lotus Seedpod Procyanidin Extract. The procyanidin extract of lotus seedpod is a light red-brown amorphous powder, and its pH value of a 1% aqueous solution is 4. The butanol/HCl hydrolysis of the water/acetone extracts gave a deep red color, which confirmed the presence of a significant amount of condensed tannins. Spectrometric quantifications showed a total polyphenol content of 90.7% according to tannin standard, and the procyanidin extract content of lotus seedpod is 98.3% on the basis of comparison with a calibration curve of grape seed procyanidin extract. On the basis of the calibration curve of procyanidin B2, the procyanidin
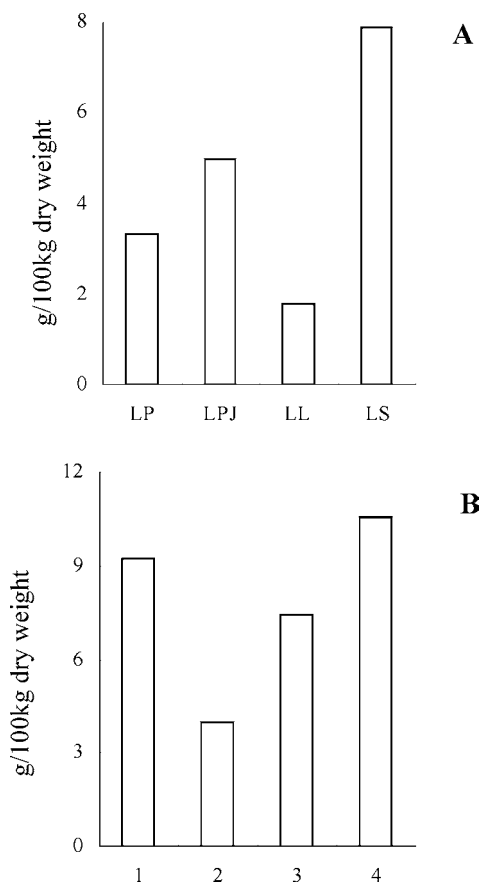
Figure 1. (**A**) Procyanidin content of different parts of *N. nucifera* Graertn. (LP, total procyanidin extract of lotus peduncle; LPJ, procyanidin extract of the joints of lotus root; LL, procyanidin extract of the lotus leaf; LS, procyanidin extract of the lotus seedpod). (**B**) Procyanidin content at different time periods of seedpod of *N. nucifera* Graertn. (**1**, July 15, 1999; 2, Aug 1, 1999; 3, Aug 16, 1999; 4, Sept 1, 1999.)
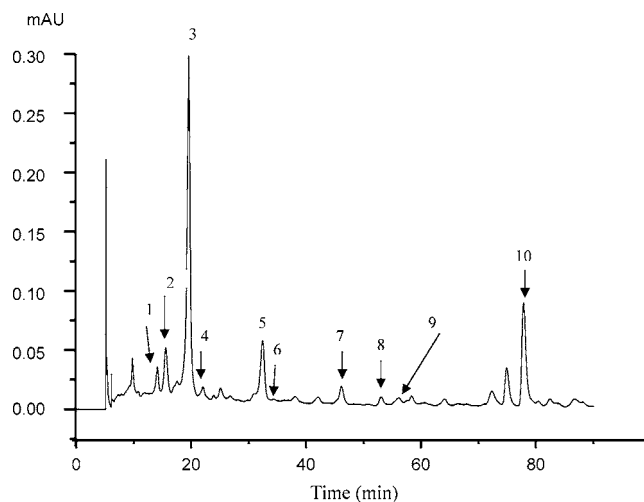

Figure 2. Separation of the procyanidin B isomers of the ethyl acetate extract of lotus seedpod procyanidins by HPLC [1, B1; 2, B3; 3, (+)-catechin; 4, B4; 5, B2; 6, B6; 7, B8; 8, B2-gallate; 9, B7; 10, epicatechin-gallate].

Table 1. Chemical Shift (δ) of Lotus Seedpod Procyanidin Extract Proton and Carbon-13

ring	proton location	δ	carbon location	δ
B			C-1	132.54
	H-2′	6.70	C-2	115.24
	H-5′	6.41	C-5	116.10
	H-6′	6.71	C-6	119.41
	3-OH	7.38	C-3	145.80
	4-OH	7.39	C-4	146.61
	5 –OH	6.79	C-4a	109.29
	7-OH	6.97	C-5, 7	158.34
			C-8a, 8a‴	157.07
			C-8a′, 8a″	157.47
A	H-6′	5.95	C-6′,6″	97.54
	H-6″	5.95	C-6	96.33
	H-8‴	5.94	C-8,8′,8″	108.29
			C-8‴	95.53
	H-2	5.86	C-2	79.80 (*cis*), 82.73 (*trans*)
	H-2′	5.92		
	H-2″	5.94	C-2′,2″	76.98 (*cis*)
	H-2‴	4.58		
C	H-3	3.83		
	H-3′	3.85	C-3′,3″	73.12
	H-3″	3.98	C-3″	73.45
	H-3‴	4.00		
	H-4′	4.01	C-3	68.70 (*cis*)
	H-4″	4.55	C-4′,4″	37.58
	H-4‴	4.57		
	H-4	2.50	C-4	29.16
	3-OH	6.77		

content of lotus seedpod extract is 63.2%. This indicated that different standards result in different results for the content of the procyanidin extract and that more work in its quantification is necessary.

Because the contents of both trimer and tetramer in the procyanidins extract were too low to be analyzed, procyanidin B was further studied in the ethyl acetate extract of lotus seedpod procyanidin extract. The different isomer contents in dimers were as follows relative to procyanidin B2: B1, 25.4%; B2, 45.7%; B3, 10.0%; B4, 4.8%; B6, 0.1; B7, 3.9%; B8, 5.0%; B2-gallate, 5.2% (**Figure 2**).

Mass spectrometric analyses were performed using an electrospray ionization (ESI) technique. ESI mass spectrometry is a soft ionization technique usually showing molecular ion peaks, making it useful for M_r determination of large biological compounds that are difficult to vaporize or ionize. Ionization was performed in the positive mode by the loss of one or several protons and formation of as many charges. The spectra were characterized by the distinct presence of several peaks at 291.1, 579.2, 731.2, 867.2, 1019.4, and 1155.3, which indicated that the main mass range of procyanidins is 290−1154. All of these signals were consistent with molecular ion peaks of polymeric catechins of the procyanidin type. Considering the M_r of a catechin unit (i.e., 290) and the loss of two protons for each interflavan linkage, m/z 291.1, 579.2, 867.2, and 1155.3 were consistent with $[M + H]^+$ of monomeric, dimeric, trimeric, and

tetrameric procyanidins, respectively, and m/z 731.2 and 1019.4 were consistent with $[M + H]^+$ of dimeric and trimeric procyanidin gallate.

The chemical shift of the procyanidins from ^{13}C NMR and ^{1}H NMR analyses is shown in **Table 1**. Catechin polymerization influences greatly the chemical shift of H-2 and H-4 in catechin, providing useful structural information. Usually, the hydrogen atoms of catechin C-2, C-3, and C-4 positions occur in the ranges 4.50−5.20 ppm (H-2), 3.74−3.99 ppm (H-3), and 2.50−3.20 ppm (H-4), respectively, in the procyanidin terminal unit. Because of catechin polymerization, the δ value of H-2 is shifted downfield to 5.20−5.97 ppm, and that of H-4 to 4.42−4.79 ppm, with aromatic ring A influenced by another catechin unit at H-4. The table shows that the δ value of H-4 is 2.50 ppm,

and there exist three signals at δ 4.01 (H-4$'$), δ 4.55 (H-4$''$), and δ 4.57 (H-4$'''$), suggesting that the flavan-3-ol units of procyanidins are linked mainly together through C(4). Moreover, the number of H-2 peaks relates to the flavan-3-ol units polymerization degree, one peak appearing downfield after catechin polymerization, indicating a dimer, two peaks indicating a trimer in the polymer, etc. (*12*). The resonance at 4.58 ppm corresponds to the procyanidin terminal H-2$'''$, 5.86 ppm corresponds to H-2, 5.92 ppm corresponds to H-2$'$, and 5.94 ppm corresponds to H-2$''$, indicating that the largest polymerization degree in the procyanidin extract is 4.

^{13}C NMR data from the analysis of the proanthocyanidin polymer preparations are shown in **Table 1**. Assignments for the resonances observed were made using those reported in the literature for model compounds and isolated polymer preparations (*13−15*). The signals at 145.80 and 146.61 ppm are therefore attributed to C-3 and C-4, respectively, in the B-ring of the procyanidin flavan-3-ol units. Flavan-3-ol polymerization influences particularly the C-2, C-3, and C-4 chemical shift of ring C and the C-6, C-8 shift of ring A. Accordingly, the resonance between 157 and 159 ppm corresponds to C-5, C-7, C-8a, C-8a$'$, C-8a$''$, and C-8a$'''$ of the aromatic A-ring of the flavan-3-ol units. Carbons in this aromatic ring at C-8 and C-6 are assigned to the signal around 96 ppm. The typical interflavanoid linkage of C-4 to C-8 is represented by the resonance at ∼107 ppm assigned to substitute C-8 (*16*). The data of the table show that the δ value of procyanidin terminal C-8$'''$ is 95.33 ppm and that the downfield signal (108.29 ppm) corresponds to C-8, -8$'$, -8$''$. This indicates that the procyanidin extract of lotus seedpod is composed of chains of flavan-3-ols units linked together through C$_4$−C$_8$ (or C$_6$) interflavanoid bonds.

The relative amounts of the stereochemical configurations (2,3-*cis* or *trans*) in lotus seedpod procyanidins extract were determined from the ^{13}C NMR spectra by integrating the resonance at 82.73 and 76.98 ppm corresponding to C-2 in 2,3-*trans* and 2,3-*cis* units, respectively. For the terminal units, a 2,3-*cis* stereochemistry was typically shown by a small resonance at 79.80 ppm corresponding to C-2, the upfield signal (68.7 ppm) corresponding to C-3, and the signal at 29.16 ppm corresponding to C-4. The hydrogen resonances of ring C in extender units are assigned as 73.12 ppm (C-3$'$), 73.45 ppm (C-3$''$), and ∼37.58 ppm (C-4$'$ and C-4$''$) and due to the influence of two aromatic rings.

Scavenging Effect of Different Concentrations of Lotus Seedpod Procyanidin Extract on Superoxide Free Radicals. The scavenging effects of lotus seedpod procyanidin extract on $O_2{}^{\bullet-}$ were expressed as IC_{50} values. The scavenging effect of the procyanidins on $O_2{}^{\bullet-}$ correlates with the concentration. The data showed that the IC_{50} value of the procyanidin extract is 17.6 mg/L, equal to that of 0.3 mg/L vitamin C. The mechanism needs to be further studied, and very likely a hydrogen/electron-transferring mechanism or a direct peroxyl radical trapping action is involved.

Scavenging Effect of Different Concentrations of Lotus Seedpod Procyanidin Extract on Hydroxyl Free Radicals. The antioxidant activity of lotus seedpod procyanidin extract can be evaluated by its ability to prevent damage of carbohydrates using 2-deoxy-D-ribose as a substrate. The scavenging effect of lotus seedpod procyanidin extract on $^{\bullet}$OH were expressed as IC_{50} value. The procyanidin extract of lotus seedpod has a scavenging effect on $^{\bullet}$OH that increases with increasing concentration. The data showed that the IC_{50} value of the procyanidin extract is 10.5 mg/L, equal to that of 4.1 mg/L vitamin C.
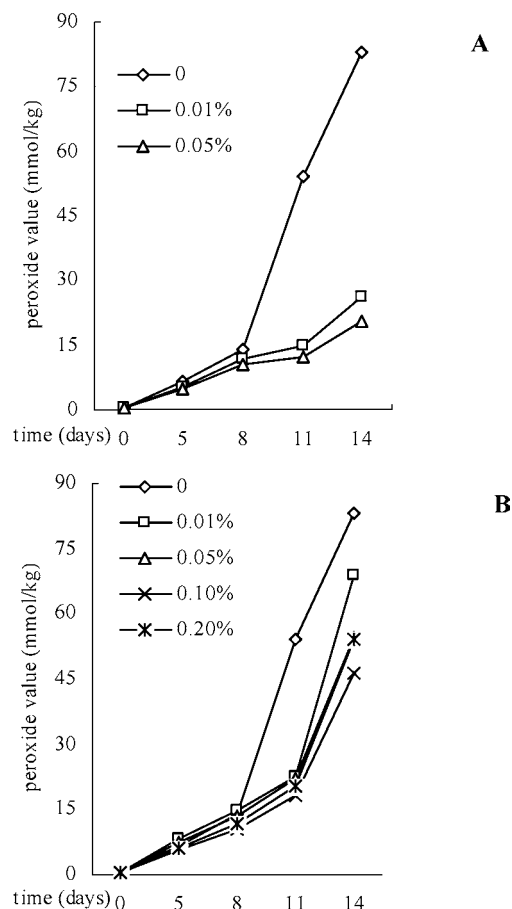

Figure 3. Effect of different concentrations of (**A**) BHT and (**B**) lotus seedpod procyanidin extract on the POV of lard oil system.

Antioxidant Activity of Lotus Seedpod Procyanidin Extract in Lard Oil System and Soybean Oil System. To determine the function of lotus seedpod procyanidin extract, the antioxidant activity of procyanidin extract in a lard oil system and soybean oil system was further investigated. The result showed that the peroxide values (POV) of the control and lard oil system containing 0.05, 0.1, and 0.2% procyanidin extract of lotus seedpod were 54, 22, 18.04, and 20 mmol/kg, respectively, after 11 days under the same conditions. This showed that 0.1% procyanidin extract had a stronger antioxidant activity than others at 60 °C and that different concentrations of the procyanidin extract had the same effect as BHT on inhibiting autoxidation of lard on the eighth day (**Figure 3**). The data indicate that the procyanidin extract of lotus seedpod could partly inhibit the autoxidation of lard.

Under the same conditions, the data showed that the procyanidin extract had a stronger antioxidant activity in a soybean oil system than in the lard system, and the antioxidant ability of 0.01−0.1% procyanidin extract was better than that of the same concentration of BHT. Furthermore, the antioxidant ability of 0.01% procyanidin extract was better than that of 0.05% BHT (**Figure 4**).

Lipid oxidation is one of the major causes of food spoilage because it leads to the development, in edible oils and fat-containing foods, of various off-flavors and off-odors. In the course of lipid autoxidation, as a typical free radical reaction, molecular oxygen abstracts hydrogen from α-methylenic groups of fatty acids and causes a free radical chain reaction. In addition, metal ions also play an important role in lipid autoxidation and accelerate peroxidation.
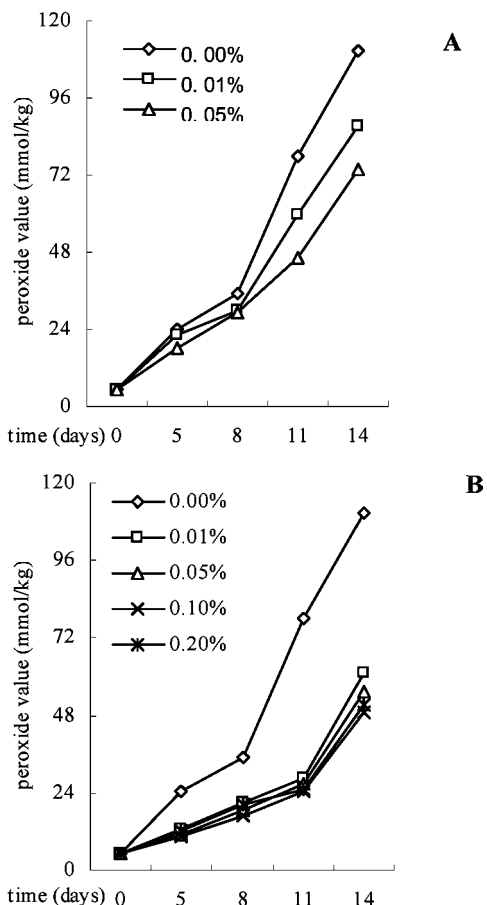
Structure and Bioactivity of Procyanidins from Lotus Seedpod

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Figure 4. Effect of different concentrations of (**A**) BHT and (**B**) lotus seedpod procyanidin extract on the POV of soybean oil system.

Procyanidins have a strong ability to chelate metal ions. Therefore, the procyanidin extract of lotus seedpod not only reacts with fatty acid free radicals to terminate the lipid free radical chain reaction but also effectively chelates metal ions so as to inhibit the lipid autoxidation.

Inhibitory Effect of Different Concentrations of Lotus Seedpod Procyanidin Extract on Lipoxygenase System. Oxidation of unsaturated fatty acids in organisms is mainly caused by lipoxygenase. The procyanidin extract of lotus seedpod effectively inhibited the lipoxygenase activity with increasing concentration. There is no significant difference in procyanidin inhibitory rate with procyanidin concentration between 62.5 and 500 μg/mL. This indicated that the procyanidin extract of lotus seedpod has a strong inhibitory effect on lipoxygenase, and whether the ability results from chelation needs further investigation.

In conclusion, for the first time, the procyanidins of nonedible parts of *N. nucifera* Gaertn. were studied. The procyanidin extract of lotus seedpod has two to four monomers that are linked through C_4–C_8 (or C_6) bonds and have strong antioxidant activity. Usually the large number of lotus seedpods, the inedible parts of *N. nucifera* Gaertn., are discarded in the countryside of China, so the study on the procyanidins of lotus seedpod will increase utilization of the region's natural resources.

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