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A-type procyanidins from *Litchi chinensis* pericarp with antioxidant activity

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Abstract

The main oligomeric procyanidins from litchi pericarp (*Litchi chinensis*) were isolated and identified. Complete assignment of epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin structure was obtained using CD, NMR and ESI-MS techniques. The known (−)-epicatechin and procyanidin A2 were also isolated from litchi pericarp. The effects of oligomeric procyanidins, A2 and epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin from litchi pericarp on free radical-scavenging were determined by using a chemiluminescent method. The results showed that all of them had a strong scavenging effect on ·OH, and the IC_{50} values were 2.60 μg/ml, 1.75 μg/ml and 1.65 μg/ml for oligomeric procyanidins, A2 and the trimeric procyanidins, respectively. The antioxidant activities of A-type dimeric and trimeric procyanidins seemed to be related to the number of hydroxyls in their molecular structures.

Keywords: Litchi; Procyanidins; Identification; Antioxidant activity

1. Introduction

Litchi is a subtropical fruit with high commercial value. Its special flavour makes it so popular in the international market. Litchi, as an economic crop, is widespread in the south of China. Since 2005, its cultivated area was more than 6×10^5 ha, and its yield was more than 1.3×10^6 ton per year. Litchi pericarp had been used as a traditional medicine with hemostatic and acesodyne functions. Those functions may due to the polyphenols of litchi pericarp. Besides anthocyanidin, there are few reports of the polyphenols of litchi pericarp. Concerning flavanols, only (−)-epicatechin and procyanidins A2 have been identified from litchi pericarp (Erwan, Thierry, Sarni-manchado, Lozano, & Cheynier, 1998; Sarni-manchado & Erwan, 2000). Therefore, the objective of this study was to purify and identify the major oligomeric procyanidins from litchi, and to determine their antioxidant activities *in vitro*.

2. Materials and methods

2.1. Materials

Fruit of litchi (*Litchi chinensis* Kwai Mi), at commercial maturation, were obtained from Conghua. The fruit arrived in the laboratory within 24 h of harvest. Fruits were peeled and the pericarp was stored at −18 °C prior to extraction.

2.2. Chemicals

Acetonitrile and acetic acid (HPLC grade) were purchased from Fisher Scientific. Toyopearl HW-40S is a product of Tosoh chemical Co. All other chemicals were of analytical grade.

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2.3. Isolation and purification of oligomeric procyanidins from litchi pericarp

Frozen litchi pericarp (50 g) was extracted using acetone (500 ml) at room temperature for 4 h. The extract was filtered through a membrane (0.45 μm), and the filtrates were concentrated under vacuum by a rotary evaporator at 40 °C. The concentrated material (50 ml) was loaded onto an ADS-17 resin column (30 cm × 2.5 cm ID), and the fraction eluted by ethanol–water (40:60, v/v) was collected. That fraction was extracted directly by ethyl acetate to obtain oligomeric procyanidins of litchi pericarp (LPOPC).

Purification of LPOPC was performed according the method described previously (Freitas & Glories, 1998). Briefly, the litchi polyphenol fraction was applied on to a Toyopearl HW-40S column (200 mm × 16 mm ID, Tosoh, Japan) and polyphenols were eluted using methanol as eluent at a flow rate 0.8 ml/min.

2.4. Butanol–HCl assay

A solution of butanol–HCl (95:5, v/v) was prepared (Haslam, 1979; Reed, McDowell, Van Soest, & Horvath, 1982). Procyanidin samples were dissolved in methanol to give a concentration of 0.1 mg/ml. Then, 1 ml of each solution was mixed with 6 ml butanol–HCl solution and 0.2 ml of iron reagent solution, i.e. $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 N hydrochloric acid (2% w/v). The solution was heated in boiling water for 40 min and then cooled to 25 °C. The procyanidin was detected at 546 nm (Porter, Hrstich, & Chan, 1986).

2.5. Reversed-phase HPLC analysis

The analysis of procyanidins of litchi pericarp by reversed-phase HPLC was performed on a Varian liquid chromatogragh, and detection was carried out using a photodiode array detector. The column used was a VP-ODS column (150 mm × 4.6 mm ID, 5 μm particle size, SHIMADZU). The method utilized a binary gradient with mobile phase, i.e. 0.4% v/v aqueous acetic acid (mobile phase A) and acetonitrile (mobile phase B). A 10 μl sample solution was injected and the elution conditions were as follows: a linear gradient from 5% to 15% B in 20 min, from 15% to 25% B in 20 min, from 25% to 35% B in 5 min, from 35% to 50% B in 5 min, and from 50% to 5% B in 5 min, at a flow rate of 1 ml/min. The column was then reequilibrated with 5% B for 5 min before the next injection. The absorbance of the eluate was monitored at 280 nm.

2.6. Mass spectrometry

For structure identification, selected samples were subjected to mass spectrometry analysis using an Agilent HPLC–MS system, which was equipped with an electrospray ionization (ESI) interface. For compatibility reasons, the mobile phases were changed to (A) 0.2% v/v aqueous acetic acid and (B) acetonitrile, and eluted with a flow rate of 0.2 ml/min. A ZORBAX Eclipse XDB-C18 (4.6 mm × 150 mm ID, Agilent) column was used and a 2 μl sample solution was injected. The elution conditions were also modified as follows: a linear gradient from 5% to 15% B in 10 min, from 15% to 20% B in 10 min, from 20% to 40% B in 20 min, from 40% to 50% B in 5 min, and from 50% to 5% B in 5 min. The column was then reequilibrated with 5% B for 10 min before the next injection. The effluent was subsequently detected by ESI-MS with a negative ion mode. The orifice voltage was −30 V and a heat capillary temperature of 275 °C. The mass scale was defined from 100 to 1200 m/z.

2.7. Circular dichroism (CD) spectrum analysis

The CD spectrum was obtained from a Jasco J-810 circular dichroism chiroptical spectrometer. The sample solution was scanned from 190 to 400 nm.

2.8. NMR analysis

For the NMR characterization of procyanidin oligomers, samples were dissolved in CD_3OD and analyzed by ^1H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectroscopy using a Varian Inova-600 instrument. Two-dimensional correlation spectra (^1H–^1H COSY, gHSQC and gHMBC) were also employed to characterize procyanidin oligomers.

2.9. Scavenging effect of oligomeric procyanidins of litchi pericarp on hydroxyl free radicals

Different concentrations of oligomeric procyanidins of litchi pericarp were tested to determine their scavenging effect on hydroxyl free radicals. Hydroxyl radical was generated by the $CuSO_4$–Phen–Vc–H_2O_2 system, as described previously (Zhang, 1994) with some modifications. Briefly, 50 μl of freshly prepared $CuSO_4$ (1.0 mM), 50 μl of phenanthroline (1 mM), 50 μl of sample under test, 700 μl of borax solution (0.05 M, pH 9.0), 100 μl of Vc solution (1 mM) and 50 μl of 0.15% H_2O_2 solution were mixed together. Chemiluminescence was measured at 37 °C using a BPCL type Ultra-weak Luminescence Analyzer made by the Institute of Biologic Physics, Chinese Academy of Sciences. The control contained all of the reagents except the sample solution, and the blank contained all of the reagents without H_2O_2. The scavenging rate was calculated as follows:

$$\text{Scavenging rate} = (CL_C - CL_S)/(CL_C - CL_0)$$

(CL_C: relative chemiluminescence intensity of control, CL_0: relative chemiluminescence intensity of blank, CL_S: relative chemiluminescence intensity of sample).

3. Results and discussion

3.1. Isolation and purification of procyanidins from litchi

The litchi extract that was only purified obtained on the ADS-17 resin column gave a positive reaction with butanol–HCl, indicating the presence of proanthocyanidins. That extract was extracted with ethyl acetate to isolate oligomeric procyanidins of litchi pericarp (LPOPC). RP-HPLC analysis of LPOPC showed numerous compounds eluting between 10 and 40 min (Fig. 1). Most of those compounds had a maximal absorbance wavelength at 277 nm, corresponding to the absorbance spectrum of flavan-3-ols. (−)-Epicatechin and (+)-catechin were identified by co-injection with standards and ESI-MS. Direct ESI-MS analysis of LPOPC extract tentatively identified A-type and B-type procyanidins from monomer to trimer from their respective mass spectra.

3.2. Identification of the main oligomeric procyanidins in litchi

Direct application of LPOPC on the Toyopearl HW-40S column allowed the separation (and purification) of procyanidins into seven fractions. Assay of the total phenols of each fraction revealed that four of those fractions had low phenolic contents. The RP-HPLC analysis of those fractions revealed few peaks of low intensity corresponding to those in Fig. 1. However, the contents of total phenols in the other three fractions were relatively high and the RP-HPLC analysis showed that the compounds in the three fractions corresponded to the main three peaks in Fig. 1. Each fraction predominantly contained a single compound. Therefore, the Toyopearl HW-40S column was shown to be very useful for purifying the main oligomeric procyanidins in litchi pericarp.

Compound 1 was identified as (−)-epicatechin by LC-ESI/MS and comparison with authentic reference. Compounds 2 and 3 were identified by ESI-MS and NMR analysis. The ESI-MS in the negative ion mode of compound 2 exhibited a signal of molecular ion ($[M-H]^-$) at m/z 575.1, which correspond to the molecular ion of B-type procyanidin dimer (m/z 577) without two protons, suggesting that it had an additional C–O–C linkage in the structure, similar to the A-type procyanidin dimer (Erwan et al., 1998). In the respective MS^2 spectrum, three fragment ions were found at m/z 449.0, 423.3 and 285.1, respectively. The fragment ion $[M-H-126]^-$ at m/z 449.0 corresponded to the elimination of a phloroglucinol molecule from the A-type dimer. The quinone methide (QM) cleavage of the interflavonoid bond produced the fragment ion at 285.1. The ion at m/z 423.3 resulted form a retro-Diels–Alder (RDA) fragmentation, with two protons less than the RDA fragment ion of a B-type dimer at m/z 425 (Maarit, Jyrki, Vladimir, & Kalevi, 2004), further indicating that compound 2 was an A-type dimer . However, all A-type dimers have the same molecular mass and similar fragment ions in the ESI-MS spectrum; the full structural identity of compound 2 was further characterized by NMR spectroscopy. Overall, the ^1H and ^{13}C NMR data of compound 2 (Table 1) were in agreement with that of procyanidin A2 (Fig. 2, I), previously identified and described (Erwan et al., 1998; Lou, Yamazaki, & Sasaki, 1999; Vivas & Glories, 1996).

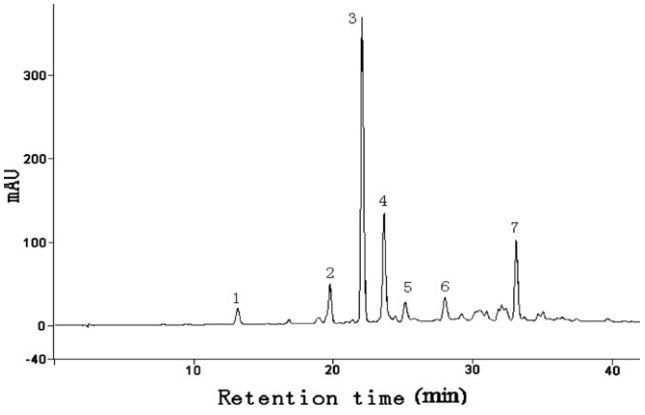

Fig. 1. RP-HPLC chromatographic profile of polyphenols of litchi pericarp, detected at 280 nm. 1: unknown compound; 2: (+)-catechin; 3: (−)-epicatechin; 4: compound 3; 5: unknown compound; 6: unknown compound; 7: compound 2.

The ESI-MS of compound 3, recorded in the negative ion mode, showed a signal of molecular ion ($[M-H]^-$) at m/z 863.1, indicating a procyanidin trimer that had an A-type interflavanoid linkage in the structure. In the respective MS^2 spectrum, a major fragment ion ($[M-H-152]^-$) was detected at m/z 711.1, resulting from the RDA fragmentation. The fragment ions from QM cleavage were also observed at m/z 285.0, 573.4 and 575.0, indicating that cleavage could take place at either the upper interflavanoid bond or the lower interflavanoid bond (Maarit et al., 2004). In the ^{13}C spectrum, the characteristic ketal carbon at δ 100.09 indicated the presence of a doubly-linked subunit. The eight upfield signals from δ 28.82 to δ 80.25 (Table 2) were attributable to the aliphatic carbons 2, 3 and 4 of each unit. The signals at δ 28.82 and δ 29.80 were attributed to the C-4 carbons of upper (u) and terminal (t) units. Consequently, the signal at δ 38.23 was due to the C-4 carbon of the middle (m) unit. These signals provide a distinct key entry point into the two-dimensional spectra (Bruyne et al., 1996). The most downfield signals from δ 145.32 to δ 157.83 were due to the phenolic carbons 5, 7, 11 and 12 of each unit, as well as the C-8a of each unit. The signals from δ 96.16 to δ 108.81 were ascribed to the carbons 6, 8 and 4a. Finally, the remaining signals, from δ 115.48 to δ 133.13, were attributed to the carbons 9, 10, 13 and 14.

In the ^1H spectrum, two signals of the highest field, at δ 2.79 and δ 2.93, were readily assigned to the H-4 of the terminal unit. The AB system at δ 3.93–4.56 (Table 2) was ascribed to the C-ring protons. The AMX system in the aromatic region from δ 5.70 to δ 6.10 was due to the H6 and H8 of each unit. In the gHSQC spectrum, H-4t at δ 2.79 correlated with C-4t at δ 28.82, indicating that the

Table 1
^{1}H NMR and ^{13}C NMR data of compound 2 isolated from litchi pericarp (600 MHz; CD_3OD)

Position	^{1}H		^{13}C	
	Upper unit	Terminal unit	Upper unit	Terminal unit
2		4.92 (br s)	100.18	81.57
3	4.05 (d, $J = 3.6$ Hz)	4.24 (m)	67.90	66.80
4	4.41 (d, $J = 3$ Hz)	2.75 (dd, $J = 2.4$, 17.4 Hz)	29.06	29.73
		2.96 (dd, $J = 5.4$,17.4 Hz)		
4a			104.05	102.21
5			156.83	156.43
6	6.01 (d, $J = 1.8$ Hz)	6.10 (br s)	98.08	96.27
7			157.94	152.12
8	6.07 (d, $J = 1.8$ Hz)		96.41	107.02
8a			154.06	151.96
9			132.26	131.02
10	7.13 (d, $J = 1.8$ Hz)	7.15 (d, $J = 1.8$ Hz)	115.41	115.46
11			146.11	146.58
12			145.47	145.81
13	6.80 (d, $J = 7.8$ Hz)	6.81 (d, $J = 7.8$ Hz)	115.82	115.73
14	7.02 (dd, $J = 2.4$, 8.4 Hz)	6.98 (dd, $J = 2.4$, 8.4 Hz)	120.16	119.58

Assignments were made by analysis of the ^{1}H–^{1}H COSY, gHSQC and gHMBC experiments.

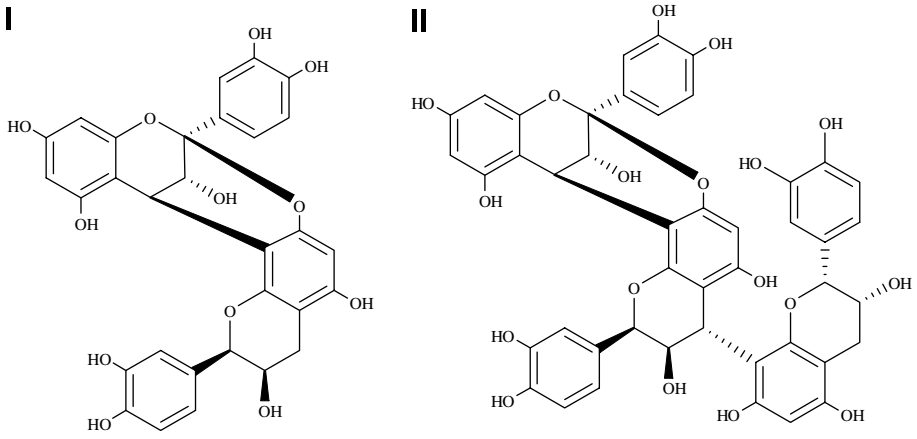

Fig. 2. Structure of procyanidin A2 (I) and procyanidin epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin (II).

Table 2
^{1}H NMR and ^{13}C NMR data for compound 3 isolated from litchi pericarp (600 MHz; CD_3OD)

Position	^{1}H			^{13}C		
	Upper unit	Middle unit	Terminal unit	Upper unit	Middle unit	Terminal unit
2		5.7 (br s)	4.38 (br s)	100.09	78.83	80.25
3	3.27 (d, $J = 3$ Hz)	4.11 (br s)	3.93 (br s)	67.49	72.58	67.13
4	4.14 (d, $J = 3$ Hz)	4.56 (br s)	2.79 (dd, $J = 6$,12 Hz)	29.80	38.23	28.82
			2.93 (dd, $J = 4.2$, 12.6 Hz)			
4a				104.91	106.35	100.17
5				156.73	151.10	155.52
6	5.96 (d, $J = 2.4$ Hz)	5.79 (br s)	6.10 (br s)	98.25	96.16	96.55
7				157.83	155.76	156.01
8	6.01 (d, $J = 1.8$ Hz)			96.41	106.74	108.81
8a				154.56	151.75	154.14
9				131.74	132.47	133.13
10	7.03 (br s)	7.31 (br s)	6.88 (d, $J = 7.2$ Hz)	116.03	116.28	116.14
11				145.75	145.44	146.59
12				145.32	145.63	146.25
13	6.82 (d, $J = 5.4$ Hz)	6.77 (d, $J = 7.8$ Hz)	6.83 (d, $J = 5.4$ Hz)	115.48	115.95	115.70
14	6.74 (d, $J = 7.8$ Hz)	7.19 (d, $J = 7.8$ Hz)	6.72 (d, $J = 7.8$ Hz)	119.87	121.31	119.42

Assignments were made by analysis of the ^{1}H–^{1}H COSY, gHSQC and gHMBC experiments.

signal at δ 29.80 was attributable to C-4u, which was directly correlated with the H-4u at δ 4.14. The proton at δ 4.56 that correlated with C-4m at δ 38.23 was assigned to the H-4m. In the ^1H–^1H COSY spectrum, the protons at δ 2.79, δ 4.14 and δ 4.56 correlated with the protons at δ 3.93, δ 3.27 and δ 4.11, respectively. Therefore, the signals at δ 3.93, δ 3.27 and δ 4.11 were from H-3t, H-3u and H-3m, respectively. Thus, gHSQC analysis revealed that the chemical shifts of C-3t, C-3u and C-3m were δ 67.13, δ 67.49 and δ 72.58, respectively.

In the gHMBC spectrum, the proton at δ 4.38 was attributed to H-2t because it was correlated with the C-3t and three other signals at δ 116.14, δ 119.42 and δ 133.13 which were assigned to the C-10t, C-14t and C-9t, respectively. In the same way, the proton at δ 5.70 was assigned to H-2m. Therefore, the chemical shift of C-2t and C-2m were δ 80.25 and δ 78.83, respectively. Furthermore, the spectrum also showed correlations between the signal at δ 6.01 and the signals at δ 157.83 (C-7u) and δ 154.56 (C-8au), permitting assignment of the signal to H-8u. The signal at δ 5.96 was assigned to H-6u, owing to correlations with the signals at δ 157.83 (C-7u) and δ 156.73 (C-5u). Similarly, the signals at δ 5.79 and δ 6.10 were assigned to H-6m and H-6t, respectively. The presence of a proton linked to the each C-6 of middle and terminal units, indicated that the C4–C8 interflavanoid linkage was the only linkage in compound 3.

The 2,3-trans and 2,3-cis configurations can be deduced from the relationship between the intensity of their C-2 signals at $\delta \sim 84$ ppm and $\delta \sim 77$ ppm (Balde, Bruyne et al., 1995; Balde et al., 1995; Rösch, Mügge, Fogliano, & Kroh, 2004). The observation of the corresponding carbon chemical shifts of the flavan C-2 carbons signalled at δ 78.83 and 80.25, were consistent with the relative 2,3-cis stereochemistry for the middle and terminal units. The chemical shifts of the C-3 carbons signalled at δ 67.49, 72.58 and 67.13, indicated that compound 3 consisted only of epicatechin units (Balde, Bruyne et al., 1995; Balde et al., 1995; Foo, Lu, Amy, & Vorsa, 2000). No proton linked to the C-2u indicated that the location of the C2–O–C7 linkage occurred in the upper two units. The two units of A-type procyanidins must possess either (2α, 4α) or (2β, 4β) double interflavanyl bonds (Lou et al., 1999). In the CD spectrum, a strong positive Cotton effect at 228 nm could be found, which reflected a 4β-flavanyl unit in the structure between the upper and middle units (Bilia, Morelli, Hamburger, & Hostettmann, 1996; Lou et al., 1999; Santos-Buelga, Kolodzieij, & Treutter, 1995), thus deciding the (2β, 4β)- configuration for compound 3. Therefore, compound 3 was assigned as epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin (Fig. 2, II).

3.3. Scavenging effect of oligomeric procyanidins of litchi pericarp on hydroxyl free radicals

The antioxidant activity of oligomeric procyanidins of litchi pericarp (LPOPC) can be evaluated by its ability to scavenge ·OH. The scavenging effect on ·OH was determined by monitoring the changes of chemiluminescence intensity of the $CuSO_4$–Phen–Vc–H_2O_2 system in the presence of LPOPC. The results showed that the scavenging effect of the extract of LPOPC on ·OH correlated with its concentration and the scavenging rate of LPOPC on ·OH could be 99.3% at a concentration of 50 μg/ml. The data showed that the IC_{50} value (concentration at which 50% of ·OH activity is inhibited) of the LPOPC extract was 2.60 μg/ml, indicating that LPOPC had a strong scavenging effect on ·OH.

In this study, antioxidant activity of the procyanidin dimer A2 and trimer epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin from litchi pericarp were determined. The relationships between the scavenging rate and the concentrations of oligomeric procyanidins (OPC), procyanidin dimer and trimer of litchi pericarp are shown in Fig. 3. The results showed that the IC_{50} values of the procyanidins on scavenging ·OH radical were 1.75 μg/ml and 1.65 μg/ml for A2 and epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin, respectively. The IC_{50} values of A2 and the trimer were 0.0030 mM and 0.0019 mM indicating that the scavenging effects of dimeric and trimeric procyanidins on ·OH depended on the number of hydroxyl groups in their molecular structures. Both the procyanidin dimer and the trimer had a much higher antioxidant capacity than had LPOPC, possibly because the latter contained some impurities or some substances with lower antioxidant activities than dimer and trimer procyanidins. For a comparison, antioxidant activity of procyanidin B3 was also determined using the same method. The results revealed that the IC_{50} value of procyanidin B3 on scavenging ·OH radical was 1.73 μg/ml, indicating that the antioxidant activity of the A-type procyanidin was lightly less strong than that of the B-type procyanidin.

In summary, HPLC–MS, NMR and CD analyses identified the main oligomeric procyanidins isolated from litchi pericarp as (−)-epicatechin, procyanidin A2 and procyanidin trimer, epicatechin-(4β→8, 2β→O→7)-epicatechin-

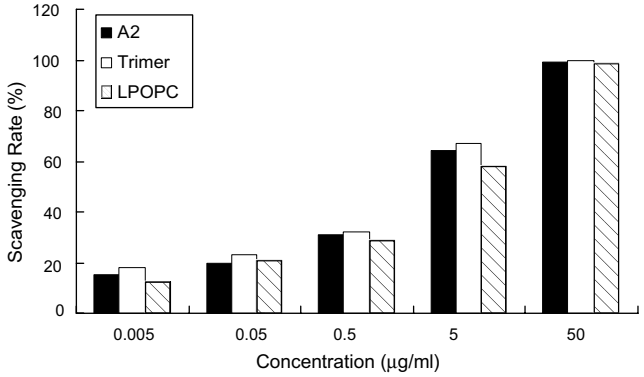

Fig. 3. Relationship between the concentrations of A2, epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatechin, LPOPC and their ability to scavenge ·OH.

(4β→8)-epicatechin. The results showed that procyanidins isolated from litchi pericarp had strong antioxidant activities *in vitro*, indicating litchi pericarp could be utilized as a new plant source of antioxidants.

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