Study on Antioxidant Activity of Catalyzed Hydrogen Degradation Product of Polymeric Proanthocyanidins (LPPC) from *Larix gmelinii* Bark

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A grading countercurrent extraction method was used to separate polymeric proanthocyanidins (LPPC) from degreased *Larix gmelinii* bark, and the purification of LPPC was performed using HP-2MGL resin. The purity of LPPC was 26.37%. The cumulative recovery rate of purified product (LPPC-1) was 98.54% by the adsorption method of HP-2MGL resin. The purity of LPPC-1 was 99.79%. Catalytic hydrogenolysis tests of LPPC-1 were performed by means of a palladium carbon catalyst. The degradation rate was 67.5%, and the residual rate was 72.1%. The results of antioxidant ability showed that the degradation product (LHOPC) had more excellent antioxidant ability compared with LPPC-1, VC, TBHQ, grape seed extract, and pine bark extract. The results, through the analysis of the linear model of MALDI-TOF/TOF MS, revealed that LHOPC was oligomeric with the tetramer as the major component and the distribution ranging from the trimer to eleven units.

Keywords: Larix gmelinii bark; Proanthocyanidins; Grading purification; Catalytic degradation; Antioxidant ability

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INTRODUCTION

Larix gmelinii grows widely in the north of China. The bark of *Larix gmelinii* accounts for 13% of the wood. Though the bark is a great treasure trove of resources, it has long been regarded as waste. In order to take full advantage of *Larix gmelinii* resources, reduce environmental pollution, and improve timber comprehensive utilization, bark research has attracted attention from researchers. The rational and effective utilization of *Larix gmelinii* bark may have a very realistic and far-reaching significance for the wood and paper industry in China.

Proanthocyanidin is one of the important activated compounds in *Larix gmelinii* bark (Zhang and Li 2001). It has a wide range of biological activity in terms of antioxidation (Ouchemoukh *et al.* 2012; Chang *et al.* 2007; Luximon-Ramma *et al.* 2005), scavenging free radicals (Bagchi *et al.* 1998; Bagchi *et al.* 2000; Virgili *et al.* 2000), anti-tumor activities (Huynh and Teel 2000; Agarwal *et al.* 2000), and cardiovascular protection and microcirculation improvement (Shao *et al.* 2009). In addition, proantho-cyanidins also have anti-inflammatory, anti-allergic (Gonçalves *et al.* 2005), anti-diarrhea (Santos *et al.* 2012), anti-aging (Tomobe *et al.* 2007), and anti-lung cancer (Sharma et al. 2010) properties, as well as the ability to enhance immunity (Tong

et al. 2011), preventing dental caries (Feghali *et al*. 2012), and other effects (Xu *et al*. 2010). In Europe, America, Asian, and some other countries, proanthocyanidins have been used in food, pharmaceutical, and cosmetic fields.

However, the content of oligomers with high utilization value is low, and the main component is LPPC, leading to reduced bioavailability and biological activity. Therefore, the catalytic degradation for LPPC becomes the urgent problem. The common degradation methods for LPPC include Pd/C catalyzed hydrogenolysis (Foo 1982) and the microbiological method (Contreras-Domínguez *et al*. 2006). Figure 1 illustrates the expected hydrogenolysis reaction. In this paper, Pd/C catalyzed hydrogenolysis was used for the degradation of LPPC. LPPC are polyphenols compounds composed of catechin, epicatechin, or gallate. LPPC are natural antioxidants and are easily degraded in acidic conditions.

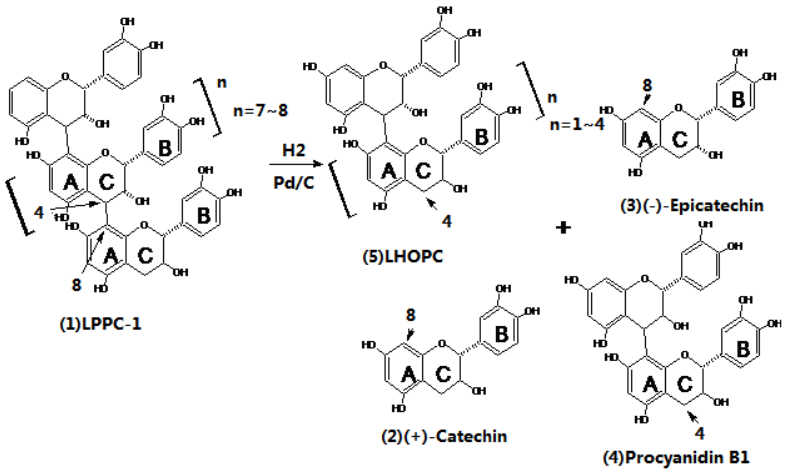

Fig. 1. Pd/C catalytic hydrogenolysis mechanism of LPPC-1

EXPERIMENTAL

Chemicals and Materials

Larix gmelinii bark was purchased from Yakeshi Tannin Factory (Inner Mongolia, China). Dried bark was powdered into a homogeneous size and then sieved (60 to 80 mesh) prior to extraction.

Proanthocyanidins reference (grape seed), grape seed extract (GS), and pine bark extract (PB) were purchased from Jianfeng Biological Products Co., Ltd., China, with a purity of 95%. Catechin, epicatechin, and procyanidins B1 standards were purchased from Chengdu Must Co., China. 10% Pd/C catalyst was obtained from Hay Star catalyst Co., China. VC, TBHQ, ABTS+•, and DPPH•, purity 98%, were purchased from SIGMA, USA. HP-2MGL and HP20 were purchased from Mitsubishi Corporation, Japan, whereas AB-8 and D101 were purchased from Resin Technology Co., Ltd., Anhui Samsung, China. The XDA-8 and LSA-10 were purchased from Xi'an Park Days adsorption material Co., China, and HB-16 was purchased from Haimin Wing Industrial Co., Ltd., China. Methanol, acetonitrile and tetrahydrofuran were chromatography grade. All the other reagents, obtained from Beijing Chemical Reagents Co. (Beijing, China), were of analytical grade. Deionized water purified by a Milli-Q water purification system from Millipore (Bedford, MA, USA) was used throughout. All solutions and samples

were filtered through a 0.45 μm nylon membrane (Guangfu Chemical Reagents Co. Tianjin, China) before injecting into the HPLC device.

Autoflex III Smartbeam MALDI-TOF/TOF MS (Bruker Corporation) was used for determining the relative molecular weight and molecular weight distribution of proanthocyanidins. The absorption wavelength of proanthocyanidins was determined by use of a TU1810 UV-Visible Spectrophotometer (Purkinje General Instrument Co., Ltd., Beijing, China). An LG10-2.4A-Speed Centrifuge was obtained from the Beijing Medical Centrifuge plant. A BP211D Electronic Balance was purchased from Mettler-Toledo Instruments Shanghai Co., Ltd., China. A JY92-2D Ultrasonic Cell Crusher was made by Xinzhike Ltd., Ning Bo, China.

Sample Preparation and Quantification

Phenolic compounds were extracted following the method described by Jiang *et al.* (2013). The lipophilic component was extracted using a supercritical CO_2 extraction device (Hu`an Supercritical Extraction Ltd. Nantong, China). Ground degreased *Larix gmelinii* bark (50.0 g) was immersed in 600 mL of 60% ethanol in a flask. Other processes were the same as the description of Jiang *et al.* (2013). The filtrate was used in the subsequent experiment; 300 mL filtrate was divided medially into three. Then, the filtrate was added in three series extraction tubes which were filled with broken ceramic discs having many small holes to increase the contact area. Ethyl acetate, 450 mL, was added to initiate grading countercurrent extraction (Yan *et al.* 2007). Filtrate was freeze-dried and ground to obtain LPPC.

Proanthocyanidins standard solution (1 mL) with different concentration (50, 101, 151, 202, and 252 μg/mL) was mixed with ferric ammonium sulfate solution (2%, 0.2 mL) and N-butanol-hydrochloric acid solution (95:5, v/v; 6 mL). The mixture was stored at 95 °C for 40 min. After reaction, the mixture was rapidly cooled down. The absorbance was measured at 546 nm, and methanol was used as control. The calibration curve was Y=0.0024X + 0.0652, (R^2=0.9893). LPPC, 20.0 mg, was dissolved in 50 mL methanol to prepare the sample solution; 1 mL sample solution was measured at the same conditions as that of standard solution above.

Purification of LPPC by Macroporous Resins

Macroporous resins were soaked with 95% ethanol for 24 h. Subsequently the macroporous resins were washed drastically with 95% ethanol, and then the ethanol was thoroughly replaced with deionized water. The pre-treated resins were dried in the drying oven at 60 °C. Pre-weighed amounts of resins were soaked in 95% ethanol and washed thoroughly with deionized water prior to adsorption experiments. One gram of dry resins and 30 mL of 10 mg/mL LPPC solution were added into 100 mL air-tight Erlenmeyer flasks. The flasks were shaken at 25 °C for 24 h (120 rpm). Then, the supernatants were discarded after adsorption equilibrium was reached, and the adsorbate-laden resins were desorbed with 30 mL 70 % ethanol. The flasks were shaken at 25 °C for 12 h (120 rpm). The purity of LPPC-1 was calculated by the formula,

$$P=w/W\times100\% \quad (1)$$

where P is the purity of LPPC-1, w is the weight of LPPC-1 (g), and W is the weight of LPPC (g).

Pd/C Catalytic Hydrogenolysis Assay

The catalytic hydrogenolysis reaction was carried out as reported previously (St-Pierre *et al*. 2013; Li 2009) and performed in a GSF-0.25 High Pressure Reactor. One gram of LPPC-1 was dissolved in 100 mL 60 % ethanol (10 mg/mL). The sample solution was mixed with 0.25 g/100 mL Pd/C. The stirring rate of catalytic hydrogenolysis reaction was 400 rpm. The reaction temperature was 100 °C, the reaction time was 3 h, and the reaction pressure was 3.5 MPa. The average degree of polymerization (DP) was calculated by the method described by Place and Maloney (1975). The degradation rate (*D*) was calculated by the following formula:

$$D = (DP_{LPPC\text{-}1}\text{-}DP_{LHOPC})/DP_{LPPC\text{-}1}\times 100\% \quad (2)$$

Analysis of HPLC and HPLC-ESI-MS

Liquid chromatography was performed with use of an Agilent1100 HPLC system (Agilent, USA) consisting of a UV-detector and a DAD-detector (Dual λ absorbance detector) with an automatic column temperature control box. A Phenomenex-C18 column (5 μm, 4.6 mm × 250 mm) was used. The concentration of catechins, epicatechin and procyanidins B1, was 1.0 mg/mL. For HPLC analysis, acetonitrile (A) - 0.5% phosphoric acid (B) was used as the mobile phase. The injection volume was 20 μL with 0.7 mL/min flow rate. The column temperature was 30 °C. The detection wavelength was 280 nm. Gradient elution was performed as follows: 0 to 45 min, 10 to 20% A (linear gradient); 45 to 60 min, 20 to 60% A (linear gradient), and 60% A for 30 min (Sánchez-Moreno *et al.* 1999).

An AB-API 2000 triple quadrupole mass spectrometer with an ESI spray source was used in negative mode to obtain MS data. MRM transitions of the compounds searched for catechins, epicatechin, procyanidins, and product ion scan MS experiments. The ion-spray voltage of ESI in negative-ion mode was set at 3500 V. The full scan mass spectra from *m*/*z* 100 to 1000 were acquired in negative ion mode with a scan speed of 1 s per scan.

Analysis of MALDI-TOF/TOF MS

A Bruker autoflex III smartbeam MALDI-TOF/TOF MS equipped with a smartbeam laser (355 nm) was used for positive ion reflectron mode spectra in the linear mode to obtain MS and MS/MS data. The accelerating voltage was 20 kV, and the duration was 200 ns. Water was used as solvent. External standards contained Angiotensin II (1046.5 Da), Bombesin (1619.8 Da), ACTHclip18~39 (2465.2 Da) and Somatostatin 28 (3147.47 Da). CsI was selected for cationization experiment. The 2,5-dihydroxy-benzoic acid was used as matrix mixed with the sample solution (1:3, V/V). 1.5 μL mixture was placed on the MALDI target.

Antioxidant Ability of LHOPC

The DPPH• radical scavenging test was determined through the method described by Gabetta *et al.* (2000). In order to estimate the IC50 value against DPPH•, 0.2 mL sample solutions at different concentration were mixed with 7.8 mL DPPH• solution (25 μg/mL). Methanol was served as control. The absorbance was measured at 517 nm. The IC50 value was defined as the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50 %.

ABTS+• (7.0 mmol/L) was mixed with potassium peroxodisulfate (2.45 mmol/L); the mixture was kept in the dark at room temperature for 14 h. The ABTS+• solution was diluted with sodium acetate solution (20 mmol/L, pH 4.5) to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Sample solution (20 μL) was added into 3 mL mixture solution. Absorbance at 734 nm was measured after reaction of 6 min. All measurements were carried out in triplicate.

RESULTS AND DISCUSSION

Purification of LPPC

Seven macroporous resins with different polarity were employed to enrich and purify LPPC, and the results are shown in Table 1. The absorption capacity and desorption ratio of LPPC on XAD-1600 and HP2MGL resins were relatively higher than those of other resins, and the results correlated with the capabilities of the resins and the chemical features of the adsorbed substance. Proanthocyanidins contain polar hydroxyl groups and non-polar benzene rings, which had affected the adsorption capacity. XAD-1600 exhibited stronger adsorption abilities than others, but lower desorption abilities. The resin HP2MGL showed both better adsorption and desorption capabilities. The purity of LPPC-1 was 99.79%.

Table 1. Comparison of Static Adsorption and Desorption Performance of Different Macroporous Resins with LPPC

Trade name	Polarity	Adsorbing capacity (mg/g)	Adsorbing ratio %	Desorption ratio %
HB-16	Non-polar	82	88	84
X-5	Non-polar	63	57	93
AB-8	Weak-polar	69	63	81
XAD-1600	Weak-polar	86	78	99
HP2MGL	Middle-Polar	94	86	94
LSA-10	Middle-Polar	95	87	83
XDA-8	polar	88	80	85

Pd/C Catalytic Hydrogenolysis of LPPC-1

For HPLC analysis, the retention time of catechin, epicatechin, procyanidin B1, and LPPC-1 was 17.3, 24.0, 13.2, and 55.5 min, respectively. The DP of LPPC-1 before and after catalytic hydrogenolysis by Pd/C was 7.99 and 2.6, respectively. The degradation rate was 67.5%, and the residual rate was 72.1%. The results in Fig. 2b demonstrated that the peak value did not appear before 50 min, indicating that there was no catechin, epicatechin, and procyanidin B1 in LPPC-1. On the contrary, signals of catechin, epicatechin, and procyanidin B1, in Fig. 2c, were detected for HPLC analysis of LHOPC. The results shown in Fig. 2 indicate that the performance of Pd/C catalytic hydrogenolysis was excellent.

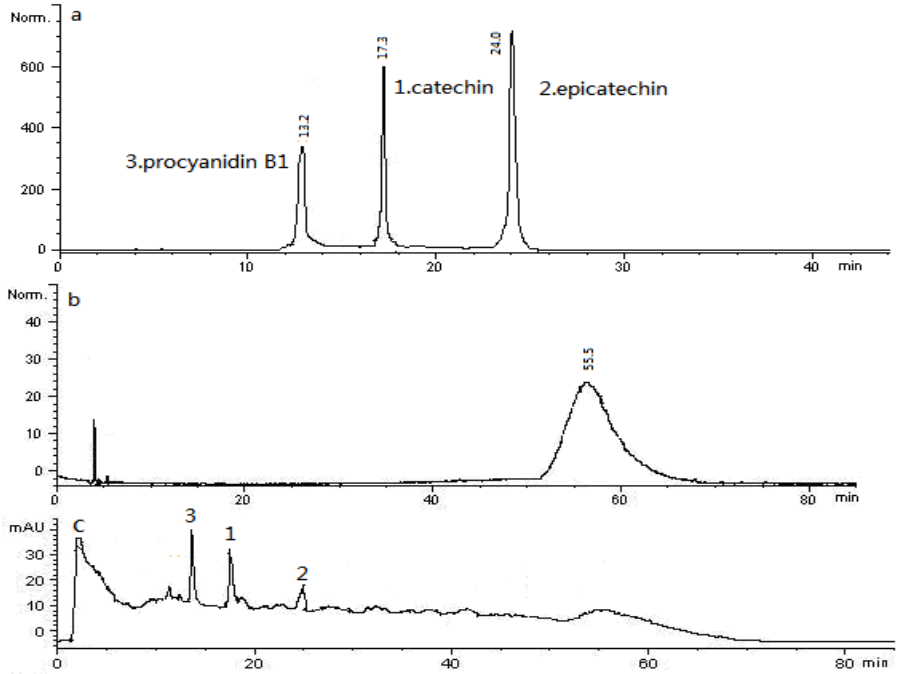

Fig. 2. HPLC analysis (a: HPLC analysis of catechin, epicatechin and procyanidin B1, b: HPLC analysis of LPPC-1, c: HPLC analysis of LHOPC)

Comparison of Catechin, Epicatechin, and Procyanidin B1 in Different Samples

In Table 2, the content of catechin and procyanidin B1 in LHOPC noticeably increased compared to those of GS and PB, and epicatechin was lower than that of GS. Procyanidin B1, which is a procyanidin dimer, was the most abundant component. Therefore, the content of procyanidin dimer affected the antioxidant activity directly. That is to say the LHOPC exhibited stronger antioxidant ability.

Table 2. Content Changes of Catechin, Epicatechin, and Procyanidin B1 after Catalytic Degradation

Component	Quality percentage (%)		
	LHOPC	GS	PB
Catechin	5.27	2.77	0.71
Epicatechin	0.31	2.34	0.10
Procyanidin B1	3.45	0.61	0.89

HPLC-ESI-MS Analysis of Catechin, Epicatechin, and Procyanidin B1 Standards

The first two parts of Fig. 3 show that the formula weights of catechin and epicatechin were both 289. As can be seen in Fig. 3c, the formula weight of procyanidin B1 was 578. These results indicated that catechin and epicatechin were monomers of procyanidine, whereas procyanidine B1 was confirmed to be the dimer structure unit of proanthocyanidins.

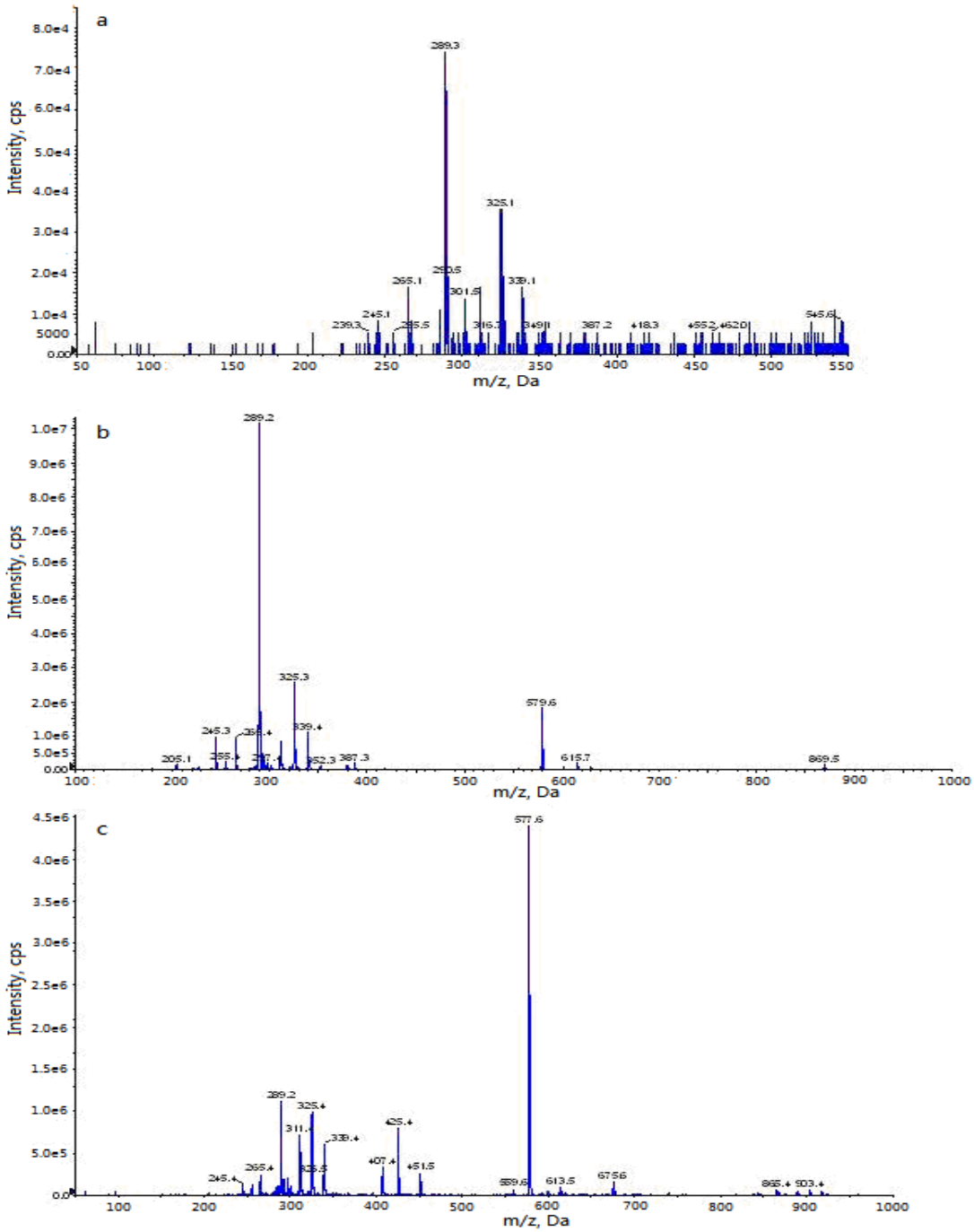

Fig. 3. HPLC-ESI-MS of catechin, epicatechin, and procyanidin B1 standards (a: HPLC-ESI-MS of Catechin standard, b: HPLC-ESI-MS of Epicatechin standard, c: HPLC-ESI-MS of Procyanidins B1 standard).

MALDI-TOF/ TOF MS Analysis of LHOPC

The structure and molecular weight distribution (MWD) of LHOPC were analyzed by MALDI-TOF/TOF MS. As can be seen in Fig. 4, LHOPC yielded signals at m/z 999.3, 1288.4, 1577.1, 1865.4, 2153.5, 2441, 2730, 3017.6, and 3305.3, respectively, when using Cs as the cationic sterically. They were separated by losses of 288 mass units, which is characteristic of EC/C losses; this seems to indicate that they were procyanidine.

These signals detected by MALDI-TOF/TOF MS can be assigned to oligomer, corresponding to procyanidin oligomers ranging from tripolymers to polymers made up of eleven monomeric units. The m/z of procyanidin was calculated from the following equation,

$$m/z = 290 + 288a + 133 \quad (3)$$

where *a* was the DP of procyanidin.

The polymeric proanthocyanidins with a DP of 3 (m/z 999.3) to 11 (m/z 3305.3) were detected in LHOPC, with most intense output corresponding to DP 4. Therefore, LHOPC was mainly comprised by the tetramer.

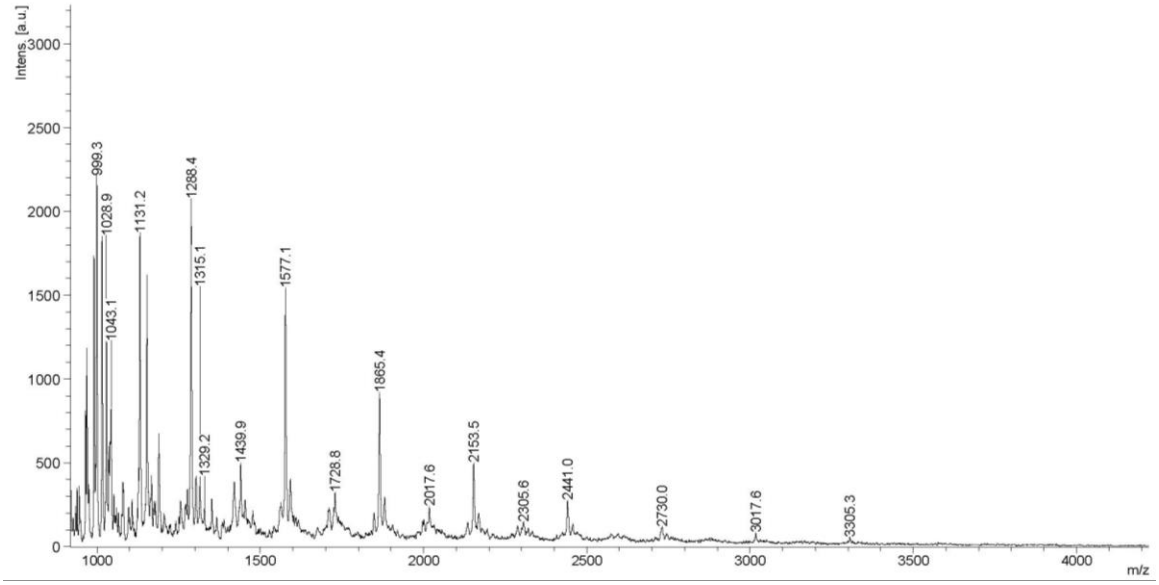

Fig. 4. MALDI-TOF/TOF MS of LHOPC

Antioxidant Ability of LPPC-1

After degradation by Pd/C, the degradation and residual rates of LPPC-1 were 67.46% and 72.1%, respectively. Within the range of 20 to 160 μg/mL, DPPH• free radical scavenging activity was augmented with the increase of sample concentration. As the concentration was raised further, the increase of DPPH• radical scavenging rate was slowed. Hydroxyl and DPPH• functions of proanthocyanidins underwent competitive binding with pheO, generating pheO-pheO so that inhibiting proanthocyanidins combined with DPPH• radical. The results shown in Fig. 5a indicated that DPPH• free radical scavenging activity of LHOPC was the strongest. As can be seen from Fig. 5b, LHOPC was found to be a very effective scavenger against ABTS+• radical compared with other samples, with the exception of GS, and its activity increased in a concentration-dependent manner. The results indicated that LHOPC has powerful antioxidant activity.

In Table 3, it can be seen that the IC50 value of LHOPC to DPPH• was lower than those of other samples, which indicated that the antioxidant ability of LHOPC was remarkable. The IC50 value of LHOPC to ABTS+• was higher than that of GS, but lower than those of others. The results of comparison of IC50 value of different samples illustrated that LHOPC possesses a strong antioxidant capacity.

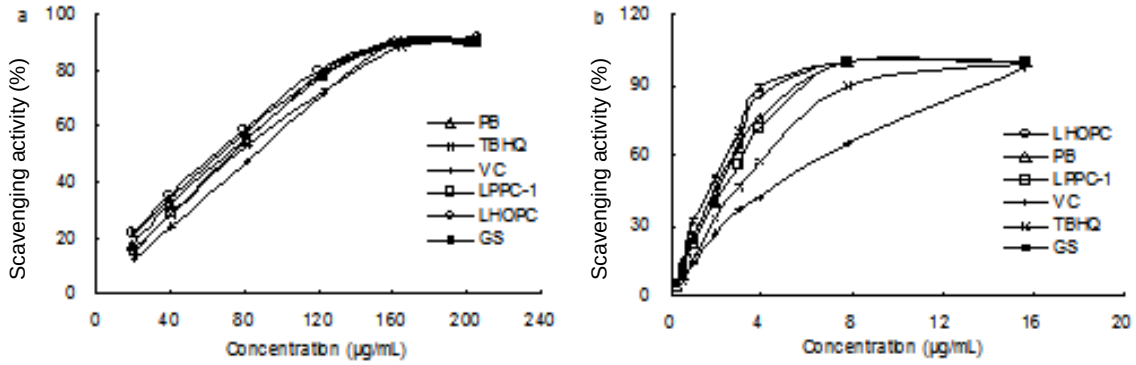

Fig. 5. Antioxidant ability of different samples (a: DPPH• radical-scavenging activity (%), b: ABTS+• radical-scavenging activity (%))

Table 3. Comparison of IC50 Values in Different Samples

Free radical	IC50 Value (μg/mL)					
	LPPC-1	LHOPC	GS	PB	TBHQ	VC
DPPH•	81.1±4.1	65.4±2.6	75.8±4.3	76.8±4.7	84.8±5.1	95.6±6.1
ABTS+•	2.7±0.12	2.3±0.11	1.9±0.12	2.5±0.23	3.2±0.31	5.5±0.35

CONCLUSIONS

1. A grading countercurrent extraction method was used to separate LPPC from *Larix gmelinii* bark, and the LPPC was purified by HP-2MGL resin. The purity of LPPC-1 was 99.79%.
2. The catalytic hydrogenolysis of LPPC-1 was carried out using Pd/C. The DP of LHOPC was 2.6. The degradation degree and residual percentage were 67.5% and 72.1%, respectively.
3. The content of procyanidine B1 analysis by HPLC after catalytic hydrogenolysis by Pd/C was increased dramatically, leading to a stronger antioxidant capacity of LHOPC.
4. This is the first time that the molecular weight distribution of LHOPC has been analyzed by the linear model of MALDI-TOF/TOF MS. LHOPC is an oligomer with trimer (m/z 999.6) to eleven oligomer (m/z 3305.3) distribution.
5. The antioxidant capacity of different samples was compared, including LPPC-1, LHOPC, GS, PB, TBHQ and VC to DPPH• and ABTS+•, the LHOPC possessed an excellent antioxidant ability.

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