

# Analysis of Purified Oligomeric Proanthocyanidins from *Larix gmelinii* Bark and the Study of Physiological Activity of the Purified Product

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A simple and efficient method for the purification of oligomeric proanthocyanidins (LOPC) from degreased *Larix gmelinii* bark was developed. The purity of LOPC was increased from 51.7% to 92.2%, and the cumulative recovery rate was 97.0%. The reversed phase high performance liquid chromatography-mass spectrum (RHPLC-MS) analysis indicated that the percentage contents of catechin (CA), epicatechin (EC), and procyanidin B1 (PB1) in purified LOPC (P-LOPC) were 5.05%, 2.02%, and 0.71%, respectively. The percentage contents of catechin and procyanidin B1 were noticeably higher than those obtained from grape seed extract (2.77% and 0.61%). The average degree of polymerization of P-LOPC was found to be 2.66. The matrix-assisted laser desorption ionization-time of flight/mass spectrum (MALDI-TOF/MS) analysis demonstrated that the dimer was the major component of purified LOPC and the distribution range was from dimer (m/z 713) to decamer (m/z 3016.6). The IC<sub>50</sub> values of P-LOPC against DPPH<sup>•</sup> (1,1-diphenyl-2-picryl-hydrazyl), OH<sup>•</sup> (hydroxy radical), and ABTS<sup>•+</sup> (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) were 76.4±3.82, 7.92±0.40, and 2.4±0.12. Purified LOPC exhibited more excellent physiological activity than VC (vitamin C), TBHQ (*tert*-butyl hydroquinone), and pine bark extract.

**Keywords:** *Larix gmelinii* bark; Oligomeric proanthocyanidins; Purification; HPLC-MS; MALDI-TOF/MS; Physiological activity

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## INTRODUCTION

*Larix gmelinii* bark contains large amounts of oligomeric proanthocyanidins (LOPC) (Zhang and Li 2001), which belong to the class of flavonoids (Fedorova *et al.* 2010). Flavonoids possess many medicinal values and are used as free radical scavengers (Yang *et al.* 2011), are protective against cardiovascular and hypertension diseases (Abir *et al.* 2007; Maria *et al.* 2014; Khan *et al.* 2014), and can be used as antioxidants (Xie *et al.* 2015; Ouchemoukh *et al.* 2012), anti-diarrhea (Santos *et al.* 2012), anti-aging (Tomobe *et al.* 2007), and anti-tumor (Vijayalakshmi *et al.* 2008; Sharma *et al.* 2010) agents. Noorafza *et al.* (2014) reported that the effects of proanthocyanidins on vascular endothelial function could reduce incidence of coronary heart disease. The study of Martinez-Micaelo *et al.* (2015) on immunomodulatory properties of procyanidin showed that the procyanidin can improve the immunostimulatory effects through the promotion

of the improvements in the ability to boost the obesity-induced weakened immune response. Therefore, proanthocyanidins are widely used in food, health care products, and the pharmaceuticals industry. High-purity products could improve the medicinal value enormously. Therefore, it is necessary to produce high-quality LOPC compounds.

At present, the main purification methods for these types of compounds include solvent extraction (Jiang *et al.* 2014a, b), nanofiltration using a membrane (Alexander *et al.* 2013; Chakrabarty *et al.* 2013), and chromatography (Ma *et al.* 2011). However, these methods present many drawbacks, such as low extraction yield, toxic solvent usage, time consuming and complex procedures, environment pollution, and high cost, which make them not suitable for food, health products, cosmetics, and pharmaceuticals industries. Therefore, it is necessary to develop a simple, efficient, and environment-friendly method for the purification of LOPC.

Because of the presence of an ideal pore structure, the availability of various surface functional groups, low cost, easy regeneration, low solvent consumption and the absence of pollution, macroporous resins have been used frequently for purification of natural products from herbal raw materials, such as biphenyl cyclooctene lignans (Ma *et al.* 2011) and podophyllotoxin (Liu *et al.* 2015). Macroporous resin has many advantages, including high physical and chemical stability, and unique selective adsorption. The other advantages constitute a closed cycle for absorption, desorption, regeneration of macroporous resins and solvent recovery, cost savings, and avoiding shortcomings caused by the use of organic solvents.

The present study aims to develop an efficient purification method for pure LOPC compounds using macroporous resins, analyze the purified LOPC from *Larix gmelinii* bark by HPLC-MS and MALDI-TOF/MS, and study the physiological activity of purified LOPC.

## EXPERIMENTAL

### Plant Materials and Chemicals

*Larix gmelinii* bark was obtained from Yakeshi Tannin Factory, Inner Mongolia, China. Before extraction, the dried bark was powdered and sieved (60- to 80-mesh) into a homogeneous mixture.

Reference proanthocyanidins (from grape seed), catechin (CA), epicatechin (EC), procyanidin B1 (PB1), grape seed extract (GS), and pine bark extract (PB) were obtained from Jianfeng Biological Products Co., Ltd., China in 95% purity. The resins AB-8 and D101 were provided by Resin Technology Co., Ltd., Anhui Samsung, China. The resins HP-2MGL and XAD7HP were purchased from Mitsubishi Corporation, Japan, and HP20 was obtained from Haimin Wing Industrial Co., Ltd., China. Resins XDA-8 and XAD-1600 were purchased from Xi'an Park Days Adsorption Material Co., China. Vitamin C (VC), *tert*-butyl hydroquinone (TBHQ), 1,1-diphenyl-2-picryl-hydrazyl (DPPH<sup>\*</sup>), and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS<sup>+</sup>) with a purity of 98% were purchased from Sigma, USA. All the other reagents were purchased from Beijing Chemical Reagents Co. (Beijing, China). Deionized water was purified by a Milli-Q water purification system from Millipore (Bedford, MA, USA) and was used throughout. All solutions and samples prepared for HPLC-MS analysis were filtered through a 0.45- $\mu$ m nylon membrane prior to use in HPLC.

### Sample Preparation and Quantification

The *Larix gmelinii* bark was degreased by supercritical fluid extraction. The sample was prepared using the procedure of Jiang *et al.* (2013) and Yan *et al.* (2007). The filtrate from extraction was freeze-dried and ground into powder form.

Ferric ammonium sulfate solution (2%, 0.2 mL) and n-butanol/hydrochloric acid solution (95:5, v/v; 6 mL) were added sequentially into proanthocyanidin standard solutions (1 mL) with various concentrations (15.8, 31.6, 63.2, 126.3, 252.5, and 505.0 µg/mL). After refluxing at 90 °C for 45 min, the mixture was cooled down rapidly. The absorption characteristics of the mixture were measured at 546 nm by a TU1810 UV-Visible Spectrophotometer (Purkinge General Instrument Co., Ltd., Beijing, China), and methanol was used as a control sample. A calibration curve was obtained corresponding to  $Y=0.0025X + 0.0532$ , ( $R^2=0.9991$ ). A good linearity was found for proanthocyanidins in the concentration range of 15.8 to 505.0 µg/mL. Oligomeric proanthocyanidins (20.0 mg) was dissolved in 50 mL of methanol to prepare the sample solution. The absorbance of the sample solution was determined under the same conditions as proanthocyanidin standard solutions.

### Purification of LOPC

The physical properties of macroporous resins used in the study are listed in Table 1. These resins were pretreated with 95% ethanol to remove the monomers and porogenic agents, washed thoroughly with deionized water to replace the ethanol, and then dried at 60 °C before use in adsorption experiments.

Static adsorption tests were performed as follows: pre-weighed amounts of hydrated adsorbent (equal to 1 g of dry resin) and 30 mL of 10 mg/mL LOPC solution were added to a 100-mL air-tight Erlenmeyer flask. The flask was immersed in a water-bath shaker (Guohua Electronics Co., Changzhou, China) at 25 °C for 24 h (at 120 rpm). Approximately 2 mL of supernatant solution was centrifuged for HPLC analysis. After being separated from the sample solution by filtration, the resin was washed with ethanol. Desorption ratio of the LOPC was measured at 546 nm.

Adsorption kinetics tests were performed as follows: pre-weighed amounts of macroporous resins (equal to 1 g of dried resin) were placed into a 100-mL air-tight Erlenmeyer flask, and 100 mL of 5% sample solution distributed in ethanol was added. The flask was shaken at 25 °C for 10 h (at 120 rpm); 2 mL of supernatant solution was centrifuged prior to HPLC at different times (0, 2, 4, 6, 8, and 10 h).

Dynamic adsorption tests were carried out as follows: a certain volume of LOPC solution (4.43 mg/mL) was loaded continuously at different feeding flow rate to a glass column (16 mm x 300 mm) wet-packed with the selected resin (Yu *et al.* 2010). The concentrations of LOPC in various collected effluents (10 mL/tube collected) were determined and the absorbing ratios of the effluents were monitored to study the effect of feeding flow rate on adsorbing capacity.

After adsorption, the laden macroporous resin was washed with deionized water, subsequently desorbed with water, and 10%, 30%, 50%, 70%, and 95% ethanol at flow rates of 1 BV/h (bed volume/hour), 2 BV/h, and 3 BV/h was used to investigate the effects of eluted velocity and volume fraction of ethanol on desorption of LOPC. The recovery of LOPC in various volume fractions of ethanol was measured to determine the optimal elution process conditions.

$$\text{Recovery} = C_1 \times 100\% / (C_2 - C_3) \quad (1)$$

where  $C_1$  is the concentration of LOPC in the eluent;  $C_2$  is the concentration of LOPC in deionized water; and  $C_3$  is the original concentration of LOPC (4.43 mg/mL).

### Analysis of P-LOPC

The composition of CA, EC, and PB1 in P-LOPC was determined using an Agilent1100 HPLC system (Agilent, USA) consisting of a UV-detector with an automatic column temperature control box. A Phenomenex-C18 column (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm) was employed for RHPLC-MS analysis. Methanol (A) - 0.4% phosphoric acid (B) was selected as the optimal mobile phase, and the gradient elution was performed as described by Sánchez-Moreno *et al.* (1999). The detection wavelength was 280 nm; the injection volume was 20  $\mu\text{L}$  with a flow rate of 0.7 mL/min; and the column temperature was 30  $^\circ\text{C}$ .

For RHPLC-MS analysis, an AB-API 2000 triple quadrupole mass spectrometer (AB Sciex Mass Spectrometry System Co., Ltd., USA) with an ESI spray source was used. Detection method was multi-reaction monitor (MRM). The electrospray voltage 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.

### Infrared Absorption Spectrum Analysis

A Fourier transform infrared spectrometer (FTIR) MAGANA560 (Thermo Nicolet Co., USA) was used to demonstrate the existence of active compounds. Three samples were dried. 2.0 mg of dried P-LOPC, GS, and PB were mixed with 200 mg of KBr to make pellets for FTIR analysis.

### MALDI-TOF/MS Analysis

The relative molecular weight and molecular weight distribution of P-LOPC were measured using a Bruker Autoflex III Smartbeam MALDI-TOF/MS (Bruker Corporation, Germany) equipped with a smartbeam laser (355 nm). An accelerating voltage of 20.0 kV was used for positive-ion mode spectra in the linear mode. The spectra were the sum of 256 shots and were calibrated with Angiotensin II (average molecular weight: 1046.5 Da), Bombesin (average molecular weight: 1619.8 Da), and ACTHclip18~39 (average molecular weight: 2465.2 Da) and Somatostatin 28 (average molecular weight: 3147.47 Da). Hydroquinonecarboxylic acid was used as a matrix and dissolved in water (10 mg/mL). Samples were dissolved in methanol (0.5 mg/mL). The matrix to sample solution ratio was 1:3 (v/v). The mixture was deposited onto the target and crystallized at room temperature. The  $m/z$  of procyanidin was calculated using the following equation,

$$m/z = 290 + 288a + 133 \quad (2)$$

where  $a$  is the average degree of polymerization (DP) of P-LOPC. The average degree of polymerization (DP) was calculated by the method described by Place and Maloney (1975).

### Physiological Activity

Samples were dissolved in methanol to prepare sample solutions in the concentration range of 10 to 200  $\mu\text{g/mL}$ .

The reduction ability of P-LOPC was then determined. For this purpose, 1 mL of various sample solutions was added to test tubes, followed by 2.5 mL of phosphate buffer (0.2 M; pH 6.6) and 2.5 mL of potassium ferricyanide solution (1%). The test tubes were immersed in a 50 °C water bath and reacted for 20 min. After the reaction, the mixture was cool down rapidly. Approximately 2.5 mL of trichloroacetic acid solution (10%) was added to the mixture and centrifuged at 4000 rpm for 10 min. The supernatant solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1%) and reacted for 10 min. The UV-Vis absorbance of the mixture was then measured at 700 nm by TU1810 ultraviolet and visible spectrophotometer.

Determination of DPPH<sup>•</sup> radical scavenging capacity was determined as follows: 0.2-mL sample solutions with various concentrations were mixed with 7.8 mL of DPPH<sup>•</sup> solution (25 µg/mL). The mixture was stored in the dark for 30 min. Methanol was used as a control sample. The UV-Vis absorbances of the sample solution and control were measured at 517 nm at various reaction times (0, 2, 5, 10, 20, and 30 min). The free radical scavenging rate for DPPH<sup>•</sup> was calculated as follows,

$$\text{Free radical scavenging rate} = (1 - [\text{DPPH}^{\bullet}]_t / [\text{DPPH}^{\bullet}]_{t=0}) \times 100\% \quad (3)$$

where  $[\text{DPPH}^{\bullet}]_{t=0}$  is the initial concentration of DPPH<sup>•</sup> at 0 min and  $[\text{DPPH}^{\bullet}]_t$  is the concentration of DPPH<sup>•</sup> at  $t$  min.

Determination of OH<sup>•</sup> radical scavenging capacity was performed as follows: (1) a solution of ortho-hydroxybenzoic acid in anhydrous ethanol (10 mM, 0.5 mL) was added into proanthocyanidins samples of methanol solution with different concentrations, and 0.5 mL of ferrous sulfate (10 mM) and 3.5 mL of distilled water was added. Then, 5 mL of hydrogen peroxide (88 mM) was added to the mixture to activate the Fenton reaction. After mixing, the absorbance (A1) of the mixture was measured at 510 nm; (2) the ferrous sulfate in (1) was replaced with 0.5 mL of distilled water, and the absorbance (A2) of the mixture was measured at 510 nm; (3) the proanthocyanidins samples of methanol solution in (1) was replaced with 0.5 mL of distilled water, and the absorbance (A3) of the mixture was measured at 510 nm. The scavenging rate ( $P$ ) for OH<sup>•</sup> was calculated as follows:

$$P = [1 - (A1 - A2) / A3] \times 100\% \quad (4)$$

Determination of ABTS<sup>+</sup> radical scavenging capacity was performed as follows: a mixture of 7.0 mM ABTS<sup>+</sup> solution and 2.45 mM potassium peroxydisulfate solution was kept in the dark at 24±2 °C for 12 to 16 h. Sodium acetate solution (20 mM, pH 4.5) was added to the mixture to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Approximately 3 mL of the above solution was mixed with 20 µL of sample solution and reacted for 6 min, and the absorbance was measured at 734 nm. The free radical scavenging rate for ABTS<sup>+</sup> was calculated using the following equation:

$$\text{Free radical scavenging rate} = (AC - AS) / AC \times 100\% \quad (5)$$

All measurements were carried out in triplicate. The IC<sub>50</sub> value could be calculated using the scavenging rates of various sample solutions with various concentrations of DPPH<sup>•</sup>, OH<sup>•</sup>, and ABTS<sup>+</sup>. The IC<sub>50</sub> value is the concentration of sample solution scavenging half of the DPPH<sup>•</sup>, OH<sup>•</sup>, and ABTS<sup>+</sup> radicals.

## RESULTS AND DISCUSSION

### Selection of the Resins

The preliminary selection of the resins was evaluated by their adsorption capacity and desorption ratios of LOPC.

In order to select the best resin for the adsorption of LOPC, adsorption and desorption capacities of LOPC on seven different resins with various polarities are calculated and listed in Table 1. The result shows that the absorption capacities of XAD-1600 (120.4 mg/g), HP2MGL (115.2 mg/g), and D101 (111.14 mg/g) were found to be higher than those of other resins, but the adsorbing ratio of XAD-1600 (68.2%) was much lower than that of HP2MGL (87.4%). For XAD-1600 (91.8%) and HP2MGL (93.4%), the desorption ratios are similar. The results shown in Table 1 indicated that HP2MGL resin exhibited the best adsorption and desorption capabilities. The adsorption and desorption characteristics of the macroporous resins correlate with their properties and chemical structure (Ma *et al.* 2009). Resins with similar polarity to target compounds exhibited better adsorption ability. The LOPC compound contains various polar hydroxyl groups and non-polar benzene rings, which will affect the adsorption capacities of the resins used. In addition, physical features of resin played an important role in processes of adsorption and desorption. The specific surface area of HP2MGL is bigger than others except that of XAD-1600. The macroporous resin HP-2MGL was also used by Li *et al.* (2006) and Li *et al.* (2009) to purify the proanthocyanidin from grape seed. And the proanthocyanidin content obtained by separation through HP-2MGL reached 96.5%. The result in this paper was similar to their report. Therefore, HP2MGL was selected for further investigation

**Table 1.** Static Adsorption and Desorption Performances of Macroporous Resins

Trade name	Polarity	Specific surface area (m <sup>2</sup> /g)	Adsorption capacity (mg/g)	Desorption ratio (%)
D101	Non-polar	≥480	111.14	89.5
HP20	Non-polar	≥600	37.3	7.5
AB-8	Weak-polar	≥480	35.9	9.9
XAD-1600	Weak-polar	≥800	120.4	91.8
HP2MGL	Middle-Polar	≥570	115.2	93.4
XAD7HP	Middle-Polar	≥380	93.9	47.9
XDA-8	polar	≥50	102.8	76.9

Data represent the mean of three replicates.

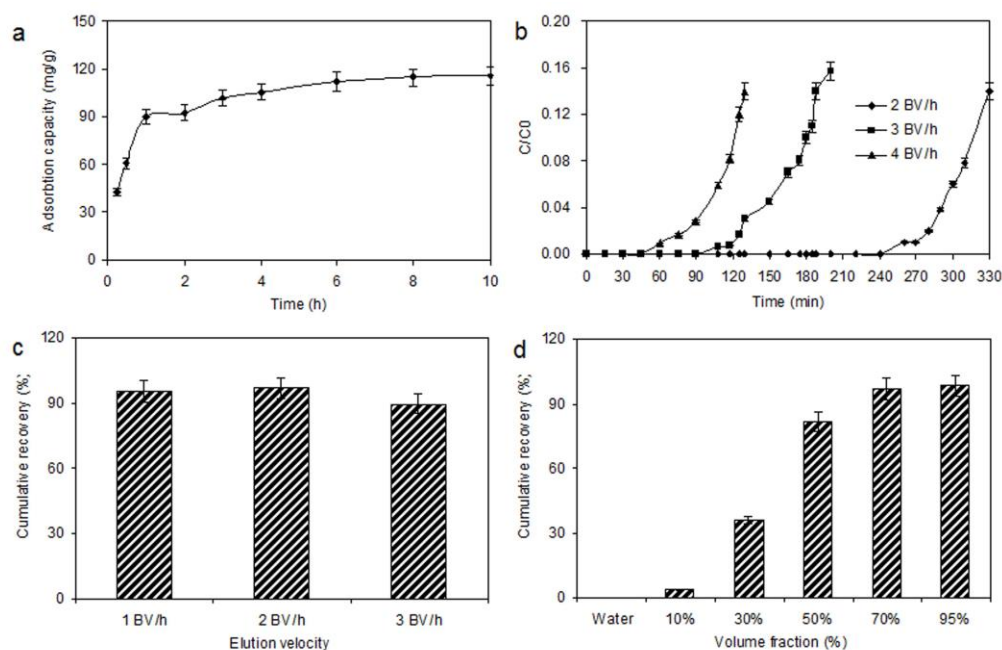
### Optimization of Purification Conditions by HP2MGL

To better understand the effect of HP2MGL resin on purification of LOPC, static adsorption and desorption experiments were carried out at 25 °C in an incubator. It can be seen in Figure 1a that the adsorption capacity of the HP2MGL resin increased rapidly during the first 1 h and reached equilibrium after 4 h.

The feeding flow rate, elution velocity, and volume fraction of ethanol were evaluated to optimize the dynamic adsorption and desorption processes using HP2MGL resin.

As illustrated in Figure 1a, the adsorption capacities of LOPC (4.43 mg/mL) increased rapidly with adsorption time before reaching the adsorption equilibrium. An asymptotic curve was reached after about 4 h of contact time, and an adsorption/desorption equilibrium was established. Therefore, 4 h was sufficient to successfully reach adsorption equilibrium over the entire system. From Figure 1b, a leaking point appeared when the concentration of LOPC in effluent reached 1/10 of the original concentration (4.43 mg/mL). With a feeding flow rate of 3 BV/h, the bed volume, absorption capacity, and feeding time were 9.1 BV, 1154.14 mg, and 182 min, respectively. The absorption capacity of LOPC decreased with high flow velocity because of the Van der Waals' force and hydrogen bonding force (Guo 2010). From overall considerations, 3 BV/h was used as a suitable feeding flow rate.

After adsorption, HP2MGL resin was washed with 70% ethanol with various elution velocities (1, 2, and 3 BV/h). Figure 1c shows that the cumulative recovery (97.01%) is the highest with a flow rate of 2 BV/h, which indicates that the vast majority of LOPC adsorbed on the resin and solid content was eluted. Therefore, the flow rate of 2 BV/h was selected for subsequent experiments.



**Fig. 1.** Purification of oligomeric proanthocyanidins (LOPC) from *Larix gmelinii* bark by HP2MGL resin: a: adsorption capacity of LOPC at various adsorption times; b: leaking point of LOPC at various feeding flow velocities; c: cumulative recovery of LOPC at various elution velocities; d: cumulative recovery of LOPC with various volume fractions of ethanol

The concentration of solvent plays the most important role in the successive desorption of adsorbates from macroporous adsorption resins (Soto *et al.* 2011). The effects of the volume fraction of ethanol (water, 10%, 30%, 50%, 70%, and 95%; v/v) on the dynamic desorption of LOPC were studied. According to the results shown in Figure 1d, different concentrations of ethanol have different effects on cumulative recovery of LOPC. This phenomenon was similar to the research reported by Fan *et al.* (2009). The cumulative recovery of LOPC was increased significantly with the increase of concentration of ethanol. It can be observed that the cumulative recovery of LOPC

reached 97.1%. The recovery had no obvious changes when feather increased the concentration of ethanol. Hence, the optimal concentration of ethanol was of 70%. The purity of LOPC purified by HP2MGL resin increased drastically from 51.72% to 92.20% (w/w) under optimal conditions (feeding flow rate of 3 BV/h; elution velocity of 2 BV/h; volume fraction of 70% ethanol). The purity of LOPC was higher than those obtained from *Elaeagnus angustifolia* L. (Sun *et al.* 2006), *Litchi chinensis* Pericarp (Zhou *et al.* 2009) and *Pinus massoniana* (Li *et al.* 2006).

### RHPLC and RHPLC-MS Analysis

The proanthocyanidins from *Larix gmelinii* bark are a mixture of catechin, epicatechin, procyanidin dimers, oligomeric proanthocyanidins, and polymeric proanthocyanidins (LPPC). The chemical composition of this is complex and has many isomers. Therefore, it is difficult to separate the chemical components by liquid chromatography. The RHPLC peak of oligomers in RHPLC is mostly within 40 min of retention time, and a big absorption peak of LPPC appeared after 40 min.

Because of the poor separation of proanthocyanidins, it is difficult to obtain a high purity of proanthocyanidins. Hence, a proanthocyanidins standard is difficult to obtain commercially. In this paper, CA, EC, and PB1 standards were used as references. Their content in P-LOPC, GS, and PB were analyzed by RHPLC.

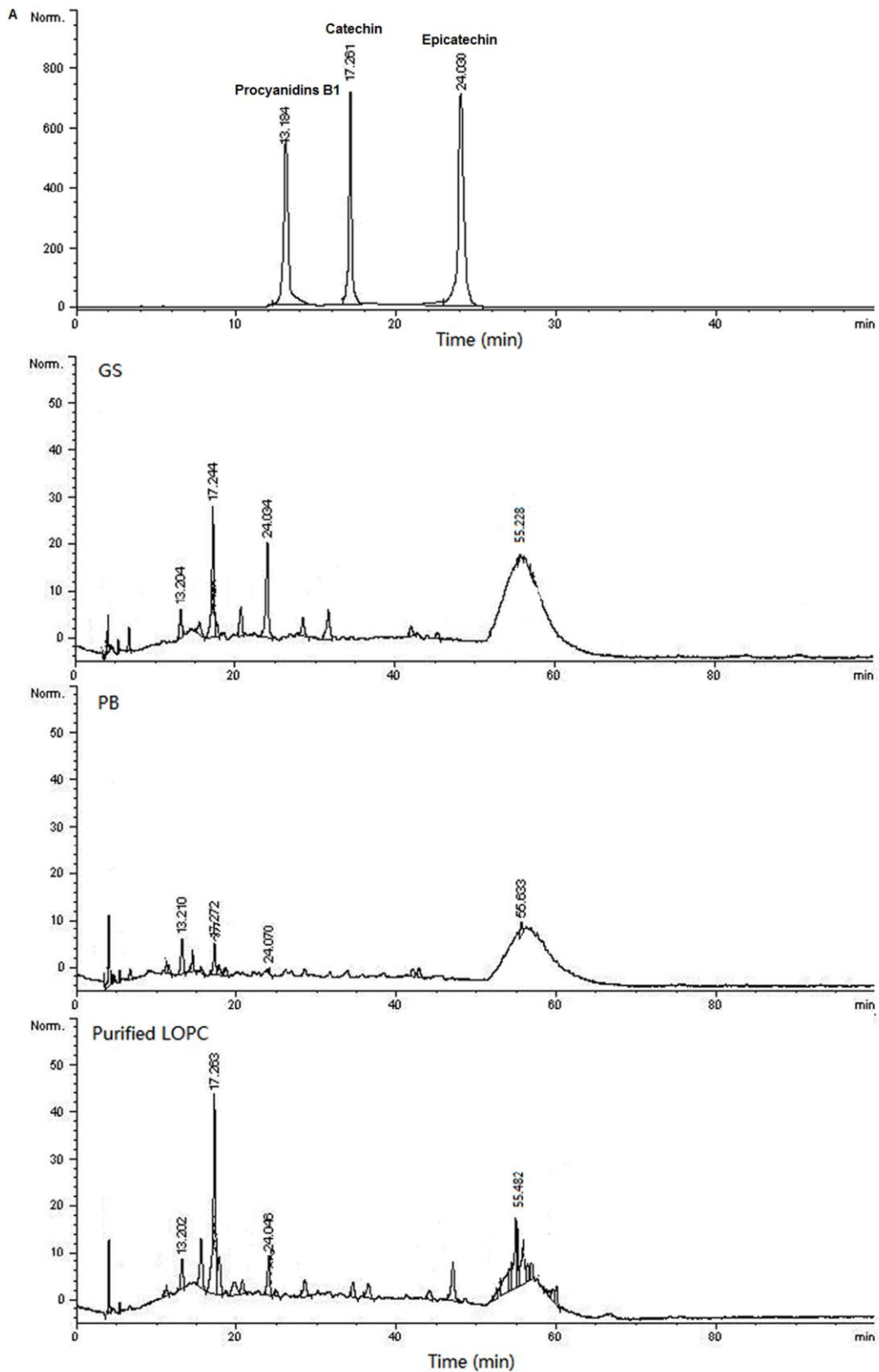
For RHPLC analysis, the concentrations of CA, EC, and PB1 were 1.0 mg/mL. It can be seen in Figure 2A that CA, EC, and PB1 could be separated very well within 45 min. The composition in GS and PB was simpler than that in P-LOPC. As can be seen in Table 2, the percentage composition (5.05%) of CA in P-LOPC was noticeably higher than those in GS (2.77%) and PB (0.71%), and PB1 (0.71%) was higher than those in GS (0.61%); the EC (2.02%) in P-LOPC was a little lower than that in GS (2.34%), but much higher than that in PB (0.10%). The procyanidin dimer PB1 was found to be the most abundant component in P-LOPC. The content of procyanidin dimer affects the antioxidant activity directly. Therefore, the P-LOPC exhibited stronger physiological activity than other tested samples.

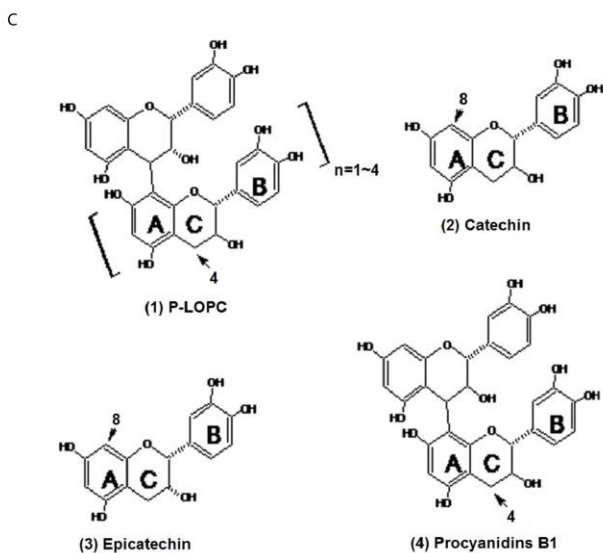
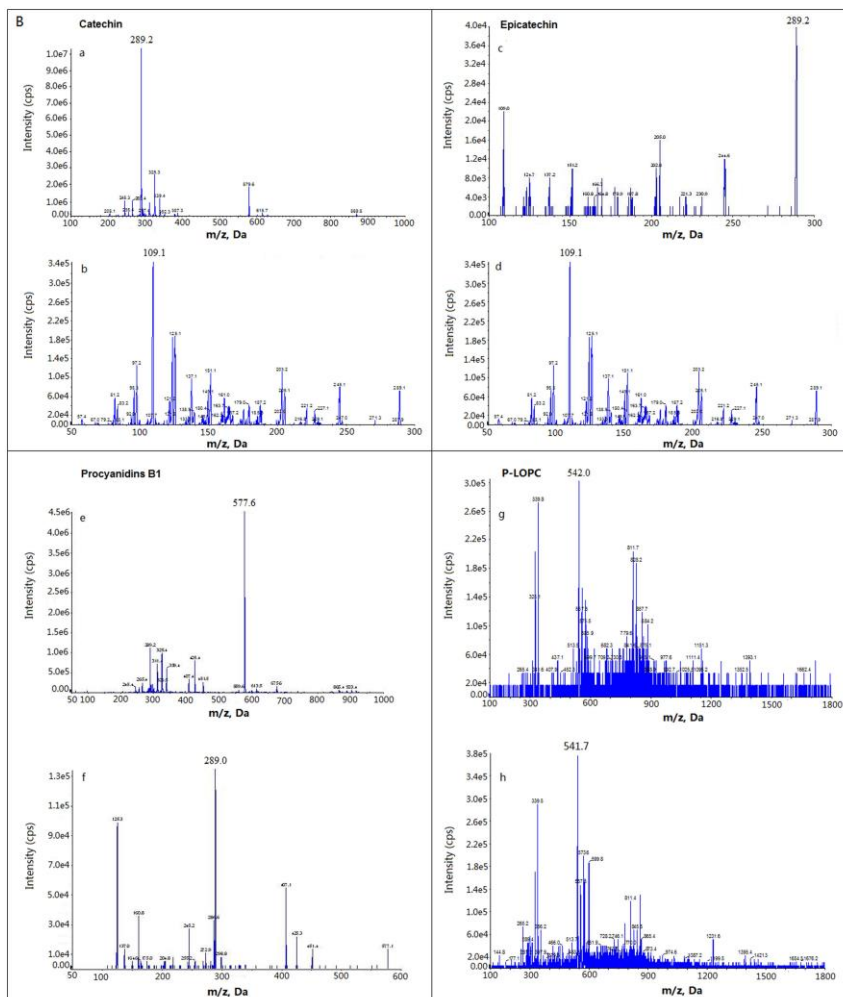
**Table 2.** Quality Percentages of CA, EC, and PB1 in Samples

Component	Quality percentage (%)		
	P-LOPC	GS	PB
CA	5.05 ± 0.25	2.77 ± 0.14	0.71 ± 0.04
EC	2.02 ± 0.10	2.34 ± 0.12	0.10 ± 0.01
PB1	0.71 ± 0.04	0.61 ± 0.03	0.89 ± 0.04

The chemical structures of CA, EC, PB1, and P-LOPC are shown in Figure 2C. As shown in Figure 2A, the retention times of the CA, EC, and PB1 standards were 17.261, 24.030, and 13.184 min, respectively. Figure 2B show the primary and secondary mass spectra of the standards, illustrating that the molecular weights of CA, EC, and PB1 are 289, 289, and 578, respectively. The secondary mass spectral fragment peaks give further proof for CA and EC as monomeric structures of proanthocyanidins and PB1 as a dimeric structure of proanthocyanidins.







**Fig. 2.** RHPLC-MS analyses. A) shows the chromatograms of catechin (CA), epicatechin (EC), procyanidin B1 (PB1), grape seed extract (GS), pine bark extract (PB), and purified LOPC (P-LOPC); B) shows the mass spectra of CA, EC, PB1, and P-LOPC; and C) shows the chemical structures of CA, EC, PB1, and P-LOPC

## FTIR

The FTIR spectra can identify the presence of certain functional groups or chemical bonds in a molecule or an interaction system. The FTIR spectra of various samples are shown in Figure 3. There is a wide and strong stretching absorption peak that belongs to phenolic hydroxyl groups at  $3390\text{ cm}^{-1}$ ; there is a stretching vibration absorption peak from C-H on methylene at  $2930\text{ cm}^{-1}$ ; the characteristic absorption peaks of benzene rings are at  $1611$ ,  $1515$ , and  $1450\text{ cm}^{-1}$ ; an ether bond stretching vibration absorption peak appears at  $1290\text{ cm}^{-1}$ ; and the characteristic absorption peak of secondary alcohols appears at  $1112\text{ cm}^{-1}$ . Therefore, the infrared structure of P-LOPC, GS and PB, demonstrated that they possess proanthocyanidins structural features.

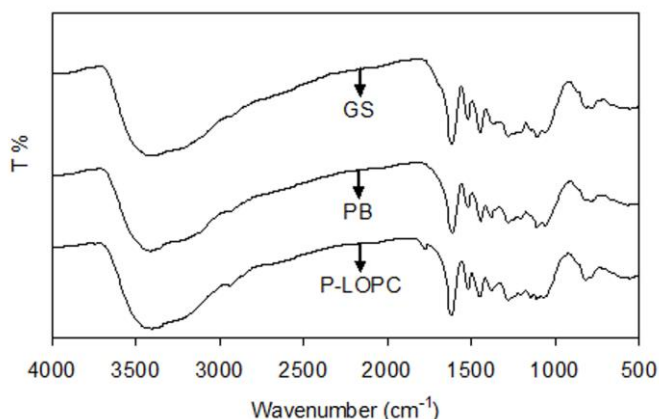


Fig. 3. FTIR spectra of P-LOPC, GS, and PB

## Molecular Weight Distribution of Purified LOPC

The MALDI-TOF/MS was used to analyze the molecular weight distribution of purified LOPC. The average degree of polymerization is 2.66. The results shown in Figure 4 indicate that signals of purified LOPC are observed at  $m/z$  713, 999.7, 1288.4, 1575.9, 1865, 2153.2, 2441.4, 2729.1, and 3016.6.

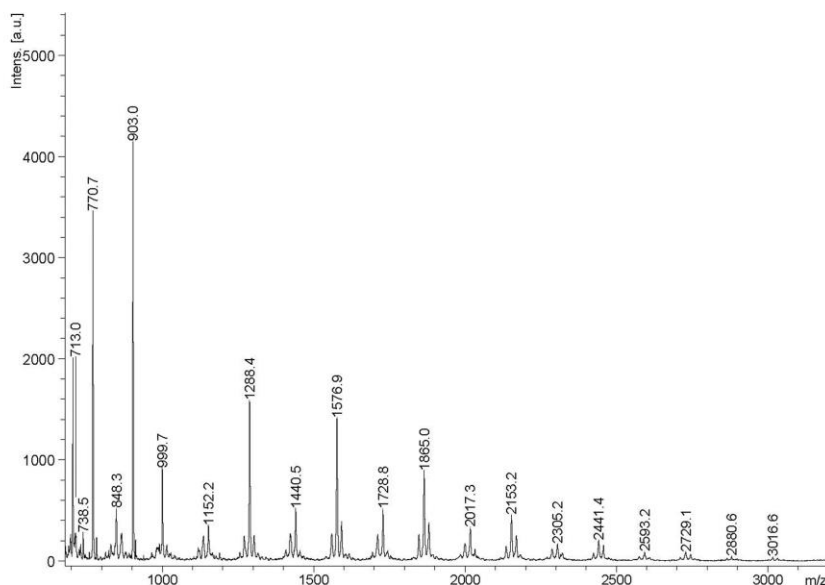


Fig. 4. The molecular weight distribution of purified LOPC

These signals are separated by losses of 288 mass units, which are characteristic of EC/C, indicating that they belong to procyanidine. These signals are assigned to oligomers, corresponding to procyanidin oligomers ranging from dimer (m/z 713) to decamer (m/z 3016.6). The signal of the dimer is the strongest, which illustrates that purified LOPC exists as a dimer

### Physiological Activity

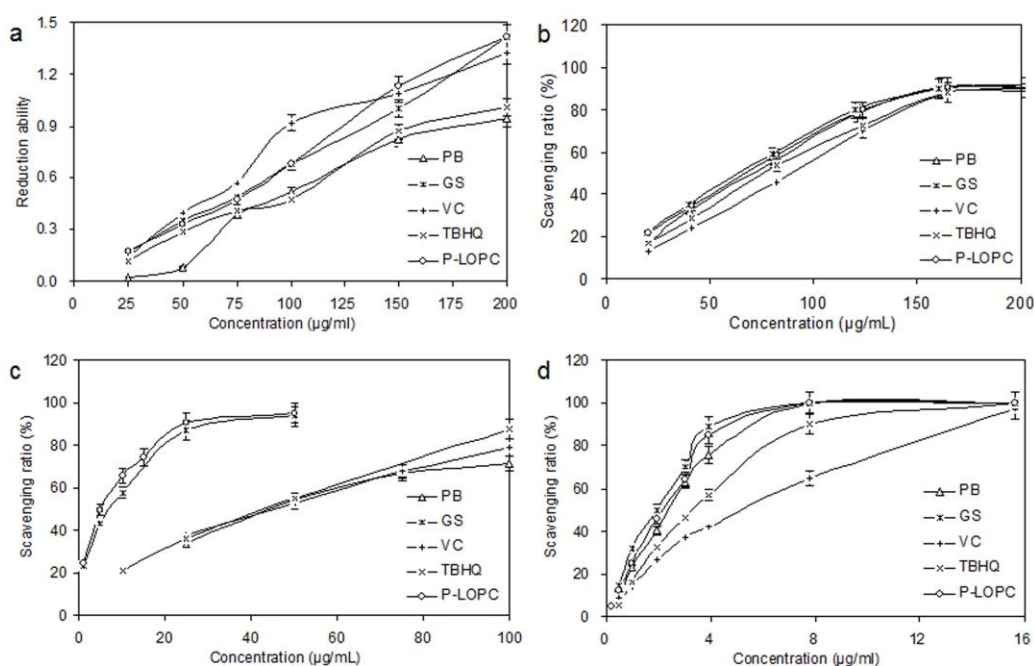
Under certain conditions, antioxidants can convert  $\text{Fe}^{3+}$  ions to  $\text{Fe}^{2+}$  ions. This reduction ability can be used indirectly to evaluate the antioxidant capability of the test substances. Also, it can be an important indicator of the potential antioxidant capacity of the test substances. Generally speaking, a component with strong reduction ability has stronger antioxidant activity. Figure 5a shows that the reduction ability of samples for hydroxyl groups is GS, P-LOPC, VC, PB, and TBHQ, in order. The reduction ability increased when the average degree of polymerization decreased, and the relative molecular mass distribution narrowed because of the steric hindrance of oligomeric proanthocyanidins. The smaller the molecular weight is, the easier hydroxyl group is to lose a hydrogen atom (Li *et al.* 2006). Therefore, P-LOPC has strong reduction ability.

The antioxidant activity of purified LOPC was evaluated by the DPPH $\cdot$  method. The purity of LOPC was 92.20% after purification by HP2MGL resin. Because of the conjugated, sterically hindered, and electron-withdrawing effect of the benzene ring, DPPH $\cdot$  is a very stable radical. In many systems, DPPH $\cdot$  can effectively capture another radical and can inhibit polymerization. Its methanol solution is purple, and the maximum absorption peak is at 517 nm. When adding a free radical scavenger, the color of the solution becomes lighter, and the absorbance at 517 nm becomes smaller. The free radical scavenging rate is calculated by absorbance to evaluate the ability of a substance to remove free radicals. Greater free radical scavenging rates show stronger antioxidant capacity. Figure 5b shows that the DPPH $\cdot$  free radical scavenging activity depends linearly on sample concentration in the range of 20 to 160  $\mu\text{g}/\text{mL}$ . The increase in DPPH $\cdot$  radical scavenging rate slowed when the sample concentration was increased further. This is because the  $\text{OH}\cdot$  and DPPH $\cdot$  of proanthocyanidins underwent competitive binding with  $\text{pheO}\cdot$ , leading to an inhibition of proanthocyanidins combination with DPPH $\cdot$  radicals. Table 3 demonstrates that the  $\text{IC}_{50}$  value of P-LOPC against DPPH $\cdot$  is lower than those of all other samples, except GS. This indicates that P-LOPC has strong DPPH $\cdot$  free radical scavenging activity.

The hydroxyl radical, which is a reduction product of ozone, is the most active, the strongest, and the most damaging of free radicals. They can cause cell necrosis or mutation. Therefore, the hydroxyl radical scavenging rate is an important indicator of the antioxidant capacity of the test substance. The results shown in Figure 5c demonstrate that there was a dose-response relationship between the free radical scavenging rate of various samples and their concentrations. The free radical scavenging rate increased when concentration was increased. The results shown in Fig. 4c indicate that the hydroxyl radical scavenging rate increased when the average degree of polymerization decreased and the relative molecular mass distribution narrowed because of the steric hindrance of oligomeric proanthocyanidins. The smaller the molecular weight of oligomeric proanthocyanidins is, the easier the hydroxyl group to lose a hydrogen atom is. Therefore, the hydroxyl radical scavenging rate is strong. According to the  $\text{IC}_{50}$  values, GS has the strongest ability to remove  $\text{OH}\cdot$  radicals, and the hydroxyl radical scavenging capacity of P-LOPC is secondary.

The ABTS<sup>+</sup> radical scavenging method is widely used for total antioxidant capacity determination of biological samples. In the reaction system, ABTS is oxidized to generate a relatively stable blue-green aqueous solution of ABTS<sup>+</sup> free radicals. After reaction between antioxidants and ABTS<sup>+</sup> radicals, the color of the reaction solution fades, and the characteristic absorbance value is decreased. The more obviously the solution fades, the stronger the antioxidant capacity of test substance is.

It can be seen from Figure 5d that there is a good linear relationship between the free radical scavenging rates of P-LOPC, GS, PB, and other references for ABTS<sup>+</sup> radicals and their concentrations. The scavenging capacity of ABTS<sup>+</sup> radicals increased when the concentration increased from 0.24 to 7.81 µg/mL. In addition to the reference VC and TBHQ, the free radical scavenging rates of other antioxidants for the ABTS<sup>+</sup> radical reached 100%.



**Fig. 5.** Physiological activity of P-LOPC: a: reduction ability of various samples; b: scavenging activity against DPPH<sup>•</sup>; c: scavenging activity against OH<sup>•</sup>; d: scavenging activity against ABTS<sup>•+</sup>

The results shown in Table 3 indicate that the differences in IC<sub>50</sub> value against DPPH<sup>•</sup>, OH<sup>•</sup>, and ABTS<sup>•+</sup> among the various samples are slight. The IC<sub>50</sub> value of purified LOPC against ABTS<sup>•+</sup> was higher than that of GS, but lower than those of PB, TBHQ, and VC, demonstrating that the physiological activity of purified LOPC is remarkable.

**Table 3.** IC<sub>50</sub> Values

Free radical	IC <sub>50</sub> (µg/mL)				
	PB	GS	VC	TBHQ	P-LOPC
DPPH <sup>•</sup>	76.65 ± 3.83	76.17 ± 3.81	97.6 ± 4.88	86.7 ± 4.83	76.02 ± 3.80
OH <sup>•</sup>	38.27 ± 1.91	6.91 ± 0.35	43.68 ± 2.18	41.27 ± 2.06	7.92 ± 0.40
ABTS <sup>•+</sup>	2.68 ± 0.11	2.26 ± 0.11	5.29 ± 0.26	3.27 ± 0.16	2.45 ± 0.12

## CONCLUSIONS

1. The oligomeric proanthocyanidins (LOPC) from *Larix gmelinii* bark purified by HP-2MGL resin revealed higher purity, low average degree of polymerization, high percentage composition of catechin and procyanidin B1. The purity of LOPC using HP-2MGL resin increased from 51.72% to 92.20%, and the cumulative recovery rate was 97.01%.
2. The percentage compositions of catechin and procyanidin B1 by RHPLC analysis were noticeably higher than those in grape seed extract, leading to a stronger antioxidant capacity of P-LOPC.
3. The infrared structure of P-LOPC was matched with the infrared structures of grape seed extract (GS) and pine bark extract (PB), demonstrating that P-LOPC, GS, and PB possess procyanidin-type structural features.
4. This is the first time that the molecular weight distribution of P-LOPC has been analyzed by the linear model of MALDI-TOF/MS. The pure LOPC was found to be an oligomer with distributions from dimer ( $m/z$  713) to decamer ( $m/z$  3016.6).
5. The antioxidant capacity of purified LOPC was higher than those of GS, PB, TBHQ, and VC to DPPH<sup>•</sup>, OH<sup>•</sup>, and ABTS<sup>•+</sup>, which indicated that P-LOPC has a stronger physiological activity.

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